Development and application of methods for planetary research

By

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Declaration

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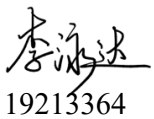
Signed: 

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List of Abbreviations

BOLD - Biological Oxidant and Life Detection Mission

BSA - Bovine serum albumin

CHA - Catalysed hairpin assembly

CBB - Coomassie Brilliant Blue

DMSO - dimethyl sulfoxide

DNA - Deoxyribonucleic acid

DTP - 4,4'-dithiopyridine

DTT - Dithiothreitol

E. coli - Escherichia coli

FAMEs - Fatty Acid Methyl Esters

GC-MS - gas chromatograph-mass spectrometer (GC-MS)

GeX - Gas-Exchange

GSH - Glutathione

HCl - Hydrochloric acid

ISCz-1 - Illite-smectite mixed layer

JSC Mars-1A - Johnson Space Centre Mars-1A Martian Regolith simulant

KGa-1b - Kaolinite

LB - Luria Bertani

LC-MS - Liquid chromatography–mass spectrometry

LD - Levenshtein distance

LR - Labelled Release

MAVEN - The Mars Atmosphere and Volatile Evolution mission

MEKC - Micellar electrokinetic chromatography

MGS-1 - Mars Global Simulant

MMS - Mojave Mars Simulant

MS - Mass spectrometry

MSL - Mars Science Laboratory

NCD - Normalized compression distance

NHD - Ninhydrin

NMR - Nuclear magnetic resonance

PAGE - Polyacrylamide gel electrophoresis

PBS - Phosphate-buffered saline

PCR - polymerase chain reaction

PL - Phospholipids

PR - Pyrolytic Release

RNA - Ribonucleic acid

SETG - Search for Extra-Terrestrial Genomes

SOLID - Signs of Life Detector

Speedvac - Speed vacuum concentrator

STx-1b - Montmorillonite

SWy-3 - Na-rich Montmorillonite

TCA – tricarboxylic acid

TCE - 2,2,2-Trichloroethanol

UV - Ultraviolet

UPLC-FLR - Ultra-Performance Liquid Chromatography with Fluorescence

XRFS - X-ray fluorescence spectrometer

2-DE - Two-dimensional gel electrophoresis

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Abstract

Explorations of extraterrestrial landscapes, primarily aimed at discerning evidence of past life and potential life-sustaining conditions, currently rely on highly specialized techniques to detect specific biomolecular signatures. Such intricate methods, which involve complex automated analytical platforms, often result in escalating mission costs and increased susceptibility to failures, including mechanical breakdowns, crash landings, vehicle losses during launch, communication breakdowns, and instrument malfunctions. There is, therefore, a growing imperative for the development of streamlined, highly specific, and definitive assays involving minimal procedural steps to bolster exploratory tests' effectiveness and mitigate associated risks.

This thesis introduces a set of solutions designed to address these challenges: single-step, ultrasensitive assays capable of detecting biomolecular indicators of life in Martian soil simulants. These assays are fine-tuned to identify an array of life signals, encompassing proteins, nucleic acids (with an emphasis on DNA), microorganisms, and metabolites such as lipids and reduced thiols. The assays' functionality and effectiveness have been verified using clays and JSC Mars-1A Martian Regolith Simulant, a simulated Martian soil sample that mimics the Martian environment.

The primary objective of this study is to augment existing detection methodologies, thereby enhancing sensitivity and simplifying assay procedures, rendering them more suitable for incorporation into space exploration missions. The findings underscore the assays' proficiency in detecting and quantifying low levels of biomolecules. These ultrasensitive assays, therefore, have the potential to significantly boost our capacity to discover life on other planets, such as Mars, and expand our understanding of life's origins within the cosmos.

Chapter 1: Introduction to the search for life signals for planetary missions

Abstract

One of the principal objectives of planetary exploration is the search for past traces of life and conditions that might support or inhibit the emergence of future life. No matter if planetary research is using telescopes or rovers and orbiters, the key point is to identify biosignatures and conditions of possible habitability in other planets. This chapter is a detailed review on the different methods applied on field missions and focusing specifically on the history of exploration of Mars, and the key findings till now. In the end, the chapter introduces the reader to the research objectives of this thesis.

1.1 Introduction

1.1.1 Astrobiology and field missions

Astrobiology was first delineated as a distinct research field by Wes Huntress from NASA in 1995 and was the interdisciplinary scientific domain dedicated to the exploration of life signals (their origin, evolution, distribution, and future) in the universe (Des Marais et al., 2008). The inaugural astrobiology roadmap from NASA presented three fundamental questions: “How does life begin and evolve? Does life exist elsewhere in the Universe? What is the future of life on Earth and beyond (Des Marais et al., 2008)?” These three questions have oriented astrobiological research towards the intricate interplay of "astro" and "bio," aiming to identify evidence that can substantiate the habitability of a planetary body. Consequently, the concept of "habitability" has emerged as a crucial objective within astrobiology, given that extraterrestrial environments within our solar system have become focal points of scientific investigation. Subsequent discoveries substantially augmented our understanding of the conditions under which early Earth evolved to become habitable (Hays et al., 2015). Under the influence of the NASA’s astrobiology map, the European AstRoMap project was launched in 2016, aiming to substantiate the proposition that life is a cosmic phenomenon (Horneck et al., 2016). This project was similar to NASA’s astrobiology map, it also accentuated the relationship among rock, water, and carbon (essential components for life) and strived to devise efficient methods to detect biosignatures.

As part of the ongoing exploration for extraterrestrial life, a diverse array of life signals is integral to the methodology of our investigative pursuits. These signals, encompassing various categories such as atmospheric gases (Snellen et al., 2013), surface morphological features (Hegde et al., 2015), specific biomolecules (Schubotz et al., 2022), discernible biosignatures (Patty et al., 2019), energy sources and gradients (Lingam and Loeb, 2019), and microbial life (Abrevaya et al., 2010), furnish indispensable evidence that could indicate the habitability of an extraplanetary body. A comprehensive overview of these categories of life signals, highlighting their respective

descriptions, methods of detection, examples, and associated limitation is presented in **Table 1.1**.

Table 1.1. Overview of Life Signal Categories in Planetary Exploration				
Life Signal	Description	Detection Methods	Examples	Limitations
Biomolecules	Organic molecules indicative of biological processes or past life	In situ measurements, mass spectrometry, chromatography, spectroscopy	Amino acids, nucleic acids, lipids, pigments, isotopic signatures	Contamination from Earth-based sources, degradation of molecules over time, challenges in distinguishing biological vs. abiotic origins
Microbial Life	Single-cell organisms that could exist in various planetary environments	Microscopy, culture-based methods, molecular biology techniques (e.g., PCR, metagenomics), biosensor technologies	Bacteria, archaea, extremophiles (e.g., thermophiles, halophiles, acidophiles, psychrophiles)	Difficulty in detecting non-Earth-like microbes, challenges in sample collection and preservation, risk of contamination
Biosignatures	Chemical, isotopic, or mineralogical features that provide evidence of biological activity	In situ measurements, mass spectrometry, chromatography, spectroscopy, microscopy	Carbon isotopic ratios, biomineralization, molecular fossils	Ambiguity in interpreting biosignatures, potential for false positives and negatives
Surface features	Physical or morphological characteristics that may indicate the presence of life or past life	Remote sensing, in situ measurements, imaging, radar mapping	Stromatolites, microbial mats, fossilized remains, chemical alteration of minerals	Non-biological processes can produce similar structures, preservation bias in the geological record
Atmospheric gases	Gases that may be indicative of biological activity or the potential for habitability	Remote sensing, in situ measurements, spectroscopy, mass spectrometry	Oxygen (O_2), Methane (CH_4), Nitrous oxide (N_2O), Sulfur gases (SO_2, H_2S)	False positives due to abiotic processes, challenges in distinguishing biological vs. geological origins
Energetics	Energy sources and gradients that can support life	Remote sensing, in situ measurements, modeling	Photosynthetically active radiation, hydrothermal activity, chemical gradients	Wide range of energy sources, life can adapt to extreme conditions, challenges in quantifying available energy

Research studies focusing on the exploration of extrasolar planets (or exoplanets) relied on the understanding of planetary size, composition, and atmospheric consistency. The common methods applied in the search of exoplanets fall into two broad categories; the direct and indirect methods. Direct methods are aiming to isolate the light reflected by exoplanets or the thermal infrared radiation emanating from the planetary surface itself, then obtain actual pictures of exoplanets to analyse their atmospheric composition. For example, researchers can detect the atmosphere of a planet by using the absorption spectroscopy. It is indicated that each gas has a slightly different absorbance wavelength, and when this happens there will be a black line on a complete spectrum. These lines (also known as Fraunhofer lines) correspond to a very specific molecule, which indicates its presence on the planet (**Figure 1.1**). By combining all the different wavelengths of lights, scientists can determine all the chemicals which are composed of the atmosphere of a planet. Indirect methods are divided into the radial velocity method, the astrometry method and the transit method and all of them depend on finding the influence of a planet on its parent star, such as the periodic variations in the parent star's brightness and colour, to deduce the existence of the planet (ESA, 2007).

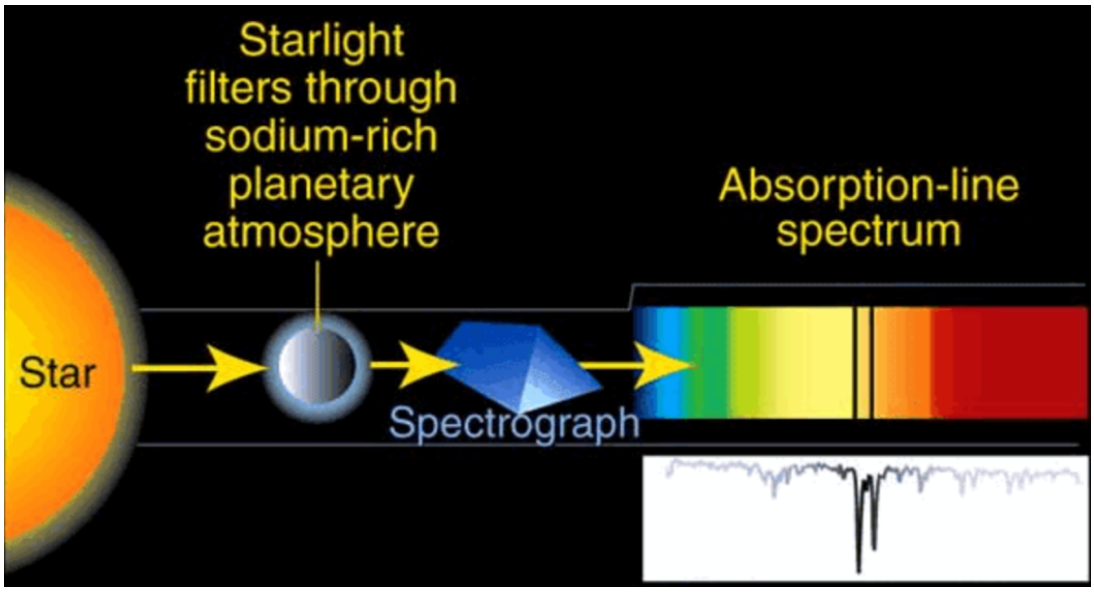


Figure 1.1. The process of light passing from a star and through the atmosphere of an exoplanet to produce Fraunhofer lines (Masetti, 2015)

The radial velocity method is the most common and effective way to detect extrasolar planets based on the radial-velocity technology by using space telescopes (ThePlanetarySociety, 2019b). It relies on the fact that a star will wobble slightly when it is orbited by a planet because of the gravitational tug. This has further led to the normal light spectrum or color signature of the star have a slight change. To be more specific, when the star is moving towards the observer, the wavelengths of the spectral lines would shift towards the blue end in the spectrum; when the star is travelling away from the observer, the opposite happens, and it will be shifted towards the red. By using highly sensitive spectrographs, astronomers can determine the mass of the planet and its orbit through the measurement of the amount of movement with time.

The astrometry method is the oldest method for planet detection to detect any wobbling of a star. It is to look for a star which is much farther away than its parent star instead of observing the planet's gravitational effects (ThePlanetarySociety, 2019a). When a star is passing in front of another from the observer's perspective, the more distant star will look temporarily brighter than normal due to the change of the gravity. After the closer star has passed, it will return to its normal brightness. This method can operate as a supplement to the radial velocity method to measure the movement of a star. However, these two methods are restricted by the atmosphere.

The transit method is to detect the decline of brightness of a star when a planet passes in front of it. The transits can block a tiny fraction of the light, astronomers can search for planets by observing the periodic changes of star's brightness. Unfortunately, only very large and gaseous planets can be detected by the transit method from the ground (ESA, 2007).

It is important to observe and research the atmospheres of exoplanets which is considered as the window into all exoplanetary properties beyond mass, radius, and orbital dynamics, and it is the most possible way to determine whether there are any life signals on exoplanets (Deming and Seager, 2017). In terms of future fields missions, the advanced facilities such as powerful ground-based telescope and space-borne spectrum are the primary instruments for the detection of exoplanets.

1.1.2 Mars exploration and probes

As a neighbour of Earth, Mars has a great value in relevance to planetary exploration. There are many reasons for exploring Mars and all of them revolve around looking for signs of life, understanding of Martian evolution and development, and making preparations for the future human exploration (ESA, 2018). Mars exploration has been achieved with several space probes send to Mars which include spacecrafts, orbiting satellites, landers and Mars Rover vehicles. The first recorded spacecraft landed on Mars happened in 1971. Soviet Union launched the Mars 3 orbiter into Martian orbit which provided the information of Martian topography, atmosphere, weather, and geology (NASA, 2019b). Later, in NASA's Mariner Mars 71 mission, the Mariner 9 orbiter provided more details about the Martian atmosphere, and it was considered as the first spacecraft to orbit around another planet (NASA, 2019a).

In the 1970s, NASA carried out the Viking Mission to Mars, which consisted of two spacecrafts, Viking 1 and Viking 2, and each of them comprised of an orbiter and a lander (NASA, 2018b). This mission aimed to capture high resolution images of Mars, reveal the structure and properties of Martian atmosphere and surface, and detect possible signals of life (NASA, 2018b). In terms of searching for evidence of life, the Viking Mission contained three types of biological experiments; the Gas-Exchange (GeX), the Pyrolytic Release (PR) and the Labelled Release (LR) experiments performed by the Viking landers on the surface of Mars. The primary experiment objectives were to detect biosignatures of microbial life from soil samples on Mars. Furthermore, there was a soil analyser equipped with an X-ray fluorescence spectrometer (XRFS) to detect the concentration of elements in samples and, most importantly, a gas chromatograph-mass spectrometer (GC-MS) specifically designed to measure biological compounds in the soil (Schuerger and Clark, 2007).

Among these experiments, the Labelled Release (LR) experiment (developed by Gilbert Levin), also known as "Gulliver", was the simplest and the most sensitive to detect microbial metabolic signatures originating from microorganisms. In this experiment, ^{14}C-labeled carbohydrates reacted with soil samples of the Martian regolith

in aqueous environment and then the released $^{14}CO_2$ gas was detected. If there were any microbes present in the soil samples, it was expected they could perform metabolic reactions with carbohydrates and release radioactive CO_2 which could be trapped on a chemically coated film at the window of a Geiger counter (Levin, 1972). The results (**Figure 1.2**) from the first LR experiment of Viking 1 presented an active cycle which heated the soil sample to 160°C for three hours prior to initiating the LR assay, and after the second injection with nutrient, soil samples reabsorbed approximately 20% of the gas product (Levin, 1997). These results indicated the possibility of life on Mars. However, there was some dispute about the LR experiment given the results from the Viking GC-MS experiment, which showed that there were no organic compounds in the Martian regolith. Another controversy was that the LR experiment was based on the assumptions that Martian microbes lived in water environment, and they could utilise the same organic nutrients to metabolize as terrestrial organisms. In general, the results from the LR experiments were inconclusive, though tantalizing (Levin, 1997).

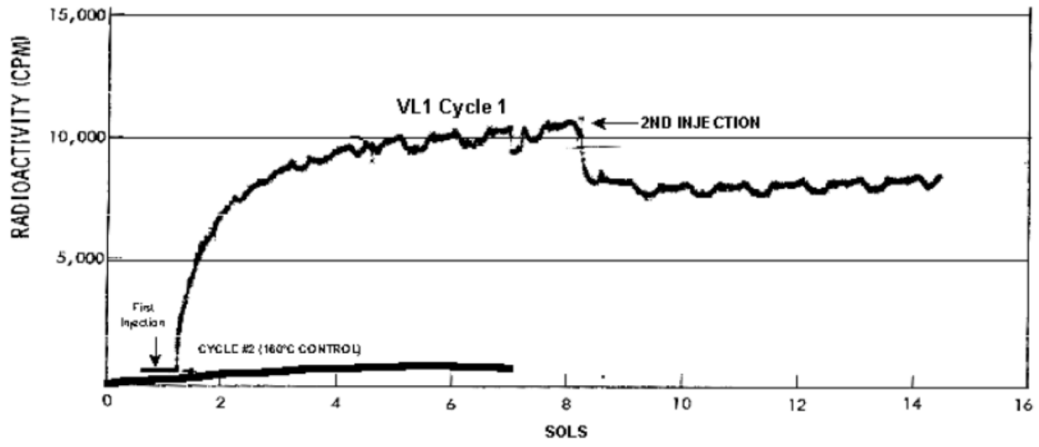


Figure 1.2. The Labeled Release (LR) experiment on Mars from VL1 (Levin, 1997).

Following, in 1996, NASA launched the Mars Pathfinder mission which contained a stationary lander and a surface rover. This mission proved that it was possible to land a free-moving rover vehicle which could travel around the surface of Mars (Williams, 2014). It is noteworthy that the surface rover, which named "Sojourner", generated a significant amount of data form Mars, including images, atmosphere, and chemical soil analyses. In the next few years, NASA had successively carried out a series of missions

about exploring Mars, such as the 2001 Mars Odyssey (Saunders et al., 2004). The mission aimed to collect data of the climate and geology of Mars and study the possibility of life existence on Mars. It also built the foundation for future missions by acting as a communications relay. In mid-2003, NASA launched two Mars Exploration Rovers: Spirit, MER-A and Opportunity, MER-B. The main mission of them is to detect if life ever arose on Mars, and to analyse the material composition of Mars to deduce if Mars could be suitable for life and prepare for human exploration of Mars (Crisp et al., 2003). The Spirit landed on the volcanic plains of Gusev crater and collected soil samples from the surface and conducted *in situ* observations. The Spirit mission confirmed the interaction of rock and water on Mars. By detecting the surface bright materials which found in olivine-bearing basalts rocks, it is suspected that these materials were caused by action of thin films of water or perhaps vapor deposition (Arvidson et al., 2006). The Opportunity landed on the Meridiani Planum region of Mars, where the rocks were mainly sandstones which supposed to be reworked by water and wind, solidified into rock, and soaked by groundwater. It showed that in the past, liquid water existed at Meridiani Planum below and occasionally at the surface. But the results also showed that the surroundings were full of arid, high ionic strength, acidic and oxidizing conditions which posed the threat to the origin of life (Squyres et al., 2006). In summary, both Spirit and Opportunity proved that in the past, wet conditions existed on Mars which possibly could have sustained microbial life.

In 2007, NASA operated the Phoenix mission to search the surface and near-surface environment of a landing site Vastitas Borealis, in the high northern area of Mars. The Phoenix lander was considered as the Mars Scout Lander, and it was focused on determining whether life ever arose on Mars and characterize the climate and geology of Mars. The Phoenix lander was equipped with a robotic arm which could dig and collect soil samples from an ice-rich layer, then these samples were transferred to the tiny ovens and a portable laboratory to analyse their chemical composition. It proved the existence of water-ice in the Martian subsurface and gave a further push to exploring the life signals on Mars. Additional findings included documenting a mildly alkaline

soil environment unlike any found by earlier Mars missions; finding small concentrations of salts that could be nutrients for life; discovering perchlorate salt, which has implications for ice and soil properties; and finding calcium carbonate, a marker of effects of liquid water (Brown et al., 2008). The last signal received from Phoenix was in November of 2008, which meant that this mission had officially ended.

Four years later, in 2011, NASA developed the Mars Science Laboratory (MSL) mission, which is also known as Curiosity. This mission was committed to explore Gale Crater on Mars to assess the habitability for microbes. In this mission, Curiosity's scientific tools discovered a series of shallow lakes, which provided the evidence of the existence of liquid water on Mars in the past. By analyzing soil samples from the "Sheepbed" mudstone in Yellowknife Bay, Curiosity detected key ingredients which are necessary for life such as sulfur, nitrogen, oxygen, phosphorus and carbon and clay minerals, all of which provided additional insight into the presence of water. In addition to these observations, Curiosity also detected organic carbon in Martian rocks and methane in the Martian atmosphere. All these evidence showed that in the past, the atmosphere of Mars was full of hydrogen, carbon, and argon and there was more water available than nowadays (NASA, 2019d). From the images collected by Curiosity from the "Teal Ridge" and "Strathdon" regions, the complicated history of water on Mars which was suspected it was not a linear process (Good and Johnson, 2019).

Following, in November 2013 MAVEN (The Mars Atmosphere and Volatile Evolution mission) launched successfully by NASA. MAVEN is designed to obtain critical measurements of the Martian upper atmosphere and ionosphere to help recognise dramatic climate change on Mars over its history. This mission provided he evidence that the major driving factor for climate change on Mars is the loss of atmosphere to space. The majority of the CO_2 on Mars has been lost to space and that makes it hard to terraform the planet by warming it, even if the CO_2 could be released and put back into the atmosphere (Jones, 2018). The MAVEN remains in operation as a communications relay and it will also provide communications support for the Mars 2020 landing (Jones, 2019). Then in 2016, European Space Agency and Roscosmos

jointly cooperate the ExoMars 2016 mission which contains the Trace Gas Orbiter and a lander called Schiaparelli. This mission is aimed to detect methane and other trace atmospheric gases which could prove the active biological or geological processes on Mars and to test key technologies in preparation for ESA's contribution to subsequent missions to Mars (ESA, 2017).

Scientists in NASA have proposed to conduct a mission for exploring Mars which include a strong and comprehensive life detection component, and it is called Biological Oxidant and Life Detection Mission (BOLD) (Schulze-Makuch et al., 2012). It will be used to search for evidence of life signals on the surface of Mars and characterize habitability of Mars. NASA has operated InSight mission to detect the interior structure and processes of Mars to understand the formation and evolution (Banerdt and Smrekar, 2019). In the coming future, NASA will develop the Mars 2020 rover mission which is designed for seeking both signs of habitable conditions and microbial life on Mars in the ancient past (NASA, 2019c). In the meanwhile, the ExoMars program will operate its 2020 mission which contains a Russian lander and an ESA rover to Mars. The rover is named after the pioneering scientist Rosalind Franklin who contributed to illuminating the double helix structure of our DNA (ESA, 2019b). The Rosalind Franklin rover equipped the PanCam cameras, a drill and the onboard laboratory, is committed to search for signs of life and it will become the first mission to have the ability to not only move across the surface but to study Martian soil at depth (ESA, 2019a, ESA, 2016). This mission will launch in the summer of 2020 and the estimated time of arrival at Mars will be in March 2021. As researchers develop a more profound understanding of Mars and more and more Mars exploration missions have been operating, it will significantly deep our knowledge of the near-surface habitability of Mars.

1.1.3 The oxidative reactivity of Martian soils

The potential existence of life beyond our terrestrial borders is inextricably linked to the integrity of organic biosignatures, which are sensitive and could be influenced by the environmental factors of their extraterrestrial abodes. One essential parameter that demands focused attention is the oxidative reactivity inherent to Martian soils, a factor of paramount significance when examining the deposition, preservation, and detection of organic biosignatures (Bak et al., 2017).

Martian soils are characterized by a distinctive oxidative reactivity, attributed to the presence of perchlorates and other oxidizing agents (Quinn et al., 2013). This oxidative environment poses a challenge, as organic biosignatures, integral to our search for extraterrestrial life, are susceptible to oxidative decomposition. This susceptibility could not only compromise the integrity of the biosignatures at their deposition sites but also affect their structural and chemical stability during water extraction processes.

Furthermore, the oxidative reactivity extends its influence on the very instruments and reagents employed in the identification of biosignatures (Georgiou et al., 2019). The chemical and structural integrity of organic reagents is paramount for the accurate detection and analysis of potential life markers. However, in an oxidative environment, these reagents might undergo alterations that could compromise the reliability of the obtained results.

In light of this, it is imperative to incorporate mitigation strategies and consider the oxidative reactivity in the development of experimental protocols for biosignature detection on Mars. Adapting and enhancing the resilience of detection assays and exploring methods to shield or stabilize biosignatures and reagents against oxidative stress could be pivotal in advancing our search for extraterrestrial life with precision.

1.1.4 Use of Soil Simulants in Life Detection Studies

A realistic approximation of planetary conditions is critical in the search for life signals, in order to adequately design and test instruments, develop exploration strategies, and

to study potential biological interactions. Soil simulants play a crucial role in this regard, providing us with a terrestrially accessible medium that closely mimics the physical and chemical properties of extraterrestrial soils.

There are various soil simulants used for planetary research in order to replicate the surface conditions of different celestial bodies. The choice of simulant depends on the specific celestial body that researchers are studying. For Mars exploration, the Johnson Space Centre Mars-1A Martian Regolith simulant (JSC Mars-1A), Mojave Mars Simulant (MMS) and Mars Global Simulant (MGS-1) are commonly used to replicate Martian conditions (Cannon et al., 2019). JSC Mars-1A is a glassy volcanic ash altered at low temperatures from Pu'u Nene, which is a cinder cone on the Island of Hawaii, MMS is developed from a volcanic ash deposit in the Mojave Desert and MGS-1 is based on the spectral and chemical data from Mars Science Laboratory and Mars Exploration Rover missions (Naz et al., 2022).

In this project, the primary soil simulant utilized is the JSC Mars-1A Martian Regolith simulant, generously provided by the NASA Johnson Space Centre. It is predominantly composed of plagioclase, complemented by lesser proportions of Ti magnetite, Ca-rich pyroxene, olivine, glassy ferric oxide particles, and trace amounts of crystalline clay minerals or phyllosilicates (Zeng et al., 2015). This mineralogical composition mirrors the observed surface conditions of Mars, thereby offering a reliable proxy for in-situ investigations. A notable attribute of the JSC Mars-1A simulant is its pH of 7.8, which falls well within the observed pH range on Mars (Fairén, 2008). Such pH equivalence bolsters the validity of the simulant and the reliability of any experimental outcomes derived from its use. The particle size of the simulant, another critical parameter, was quantified using a particle size analyzer. The findings revealed a mean particle size of approximately 150 microns. This size is coherent with the particulate size range seen in the Martian soil, which spans from fine dust to coarse sand (Oravec et al., 2021). While the humic and moisture content of the simulant was not directly assessed in this study, it is important to note that both are generally considered to be low in Martian soil due to the absence of organic matter and water on the planet's surface (Qureshi, 2023).

In addition to the JSC Mars-1A simulant, a variety of clay minerals were employed in this project as well as they have been identified in multiple locales, signalling past aqueous activity – a pivotal aspect for the potential harbouring of life (Tu et al., 2021). These minerals, characterized by their fine-grained, layered silicate structures, offer a complex array of biochemical interactions and environmental narratives that can unveil crucial insights into the potential habitability on Martian subsurface. The significance of clay minerals is accentuated by their unique structural attributes. Intercalation and swellable properties, two inherent characteristics of these minerals, make them a focal point in planetary missions for searching life signals (Bosak et al., 2021). Their capability to integrate and retain molecules within their structural layers and expand upon hydration underscores their potential as micro-environments that may harbor and protect organic compounds and other biosignatures (Fomina and Skorochod, 2020). The protective capacity of clay minerals is primarily attributed to their layered configuration, offering sanctuary to organic moieties from the harsh surface conditions of Mars, such as intense radiation and oxidative chemicals. The encapsulation of organic molecules within these layers not only ensures their preservation but also fosters conditions where prebiotic chemistry and life itself may emerge and sustain (Kloprogge and Hartman, 2022). Specifically, smectites and kaolinite, two types of clay minerals identified on Mars, warrant distinct attention. Smectites are renowned for their ability to expand upon hydration, indicative of their role in past aqueous activities and potentially, in supporting life (Azua-Bustos et al., 2020). Kaolinite, which holds significant interest in Mars studies due to their formation conditions. Their presence on Mars suggests extreme water movement or acidity levels that result in extensive cation reduction in the parent rock (Cuadros et al., 2019). Analytical techniques such as Fourier Transform Infrared Spectroscopy (FTIR) and X-Ray Diffraction (XRD) play a pivotal role in deciphering the hidden stories embedded within these clay minerals (Petit, 2006). FTIR provides insights into the functional groups of organic molecules nested within the clays, revealing their chemical composition and potential biological origins. Concurrently, XRD offers precise depictions of molecular arrangements and structural alterations, delineating the intercalation of organic entities within the clay's

layers.

In the course of this study, the employment of standardized soil simulants emerged as a fundamental component for appraising interactions with various biosignature analogues. However, an intrinsic limitation became apparent - the non-activated energy state of these simulants. This shortfall is not exclusive to the current study but extends to the broader landscape of astrobiological research. The non-activated state potentially compromises the authenticity of the simulants, diminishing their congruence with the highly reactive conditions characteristic of natural extra-terrestrial environments, including the Martian soil.

Addressing this gap calls for an intensified approach to augmenting the fidelity of soil simulants, ensuring they encapsulate the intricate chemical dynamics inherent to extra-terrestrial terrains. A prospective strategy is the fortification of these simulants with specific reactive agents. For instance, the integration of metal hypochlorite could serve to represent reactive chlorine species effectively, while the inclusion of metal peroxides and superoxides stands poised to emulate the role of reactive oxygen species generators.

This enhanced simulation environment, enriched with reactive agents, is anticipated to provide a more accurate representation of the oxidative and reactivity profiles inherent to extra-terrestrial soils. Such advancements stand to amplify our insights into the robustness and detectability of a spectrum of biosignature analogues, spanning proteins, DNA, amino acids, lipids, and bacteria. The potential to examine these entities under conditions echoing the authenticity of extra-terrestrial terrains elevates the precision of our investigative outcomes.

The proposed refinement in soil simulant employment extends beyond mitigating existing limitations; it heralds an era of enriched precision and nuanced understanding in astrobiological investigations. It epitomizes the evolutionary trajectory of astrobiological methodologies, aligning them with the multifaceted and dynamic characteristics of planetary soils, and thereby enhancing the depth and breadth of our extra-terrestrial exploratory initiatives.

1.1.5 Modifications for planetary research and the engineering challenge

As it is obvious from the aforementioned, planetary exploration missions have focused on the search for organic matter and liquid water, but none of them is a clear indicator of past or present life and the results obtained by the exobiological experiments such as the one carried out by the Viking missions which did not allow to definitely conclude the presence of biological activity on Mars. This indicates that numerous challenges still exist. The first and foremost challenge is how to fly off Earth. For space exploration, escaping the tremendous gravity of the Earth is a key factor. According to calculations, if an object on Earth's surface wants to fly free, it needs to shoot up and out at speeds exceeding 25,000 mph which result in significant cost in terms of money to support this instrumentation. NASA spent nearly $200 million just to launch the Mars Curiosity rover, which did not include the other expenses such as the daily supplies of the crew in the manned aircraft (NASA, 2012). To achieve the speed, using composite materials like exotic-metal alloys and fibered sheets to reduce the weight and combining with fuel mixtures, the aircraft can get away from Earth's gravity. Finding efficient and powerful propellants is also a challenge for rocket research experts. In addition, space debris is becoming a problem that human beings cannot ignore in space. Various types of debris, such as orbital debris and tracking debris in space can threaten the normal operation of spacecraft. They can also increase the potential danger to the International Space Station, space shuttles and other spacecraft with humans aboard (NASA, 2013).

Besides, building and launching rover missions are enormous and expensive. It is reported that NASA's next 2020 Mars rover will cost $2.46 billion (Voosen, 2019). Due to the technical issues, the cost for rover missions is gradually growing. In this case, developing small independent platforms could be inexpensive solutions such as CubeSats. CubeSats are designed as the research spacecraft called nanosatellites which could apply in aspects of science, exploration, technology development, education or operations (NASA, 2018a). It is also considered as a low-cost technology compared to traditional satellites. The low-cost of launching CubeSats reflects in two aspects. On the one hand, a rocket doesn't need much fuel to heft them due to their light weight. On

the other hand, they can share a rocket with a larger satellite, making it possible to get to space on the coattails of the heavier payload (Howell, 2018).

1.1.6 Research objectives and thesis structure

The quest for life signals has become the cornerstone of exoplanet missions, propelling the development of increasingly advanced technologies for identifying biomarkers and inhibitors. The central assumption of this thesis rests on the assumption that extra-terrestrial life, if it exists, is composed of organic constituents akin to those found in terrestrial organisms and follows similar biochemical processes. This supposition underpins the methodologies proposed for detecting biosignatures analogous to those of Earth's life forms. It's crucial to acknowledge the speculative nature of this assumption, given the current lack of empirical data on extra-terrestrial biochemistry.

The premise that life, regardless of its planetary origin, is anchored in carbon-based molecules is not unfounded but is, rather, a conjecture rooted in the universal functionalities observed in Earth's diverse biota. Several foundational principles underscore this hypothesis. First, the propensity for catalytic activities is considered ubiquitous, facilitating rapid biochemical reactions essential for life's dynamism. Second, the containment of these reactions within defined spatial boundaries is deemed critical to mitigate the dilution of essential substrates and products, ensuring the persistence of metabolic activities vital for life's sustenance.

Additionally, the role of water as the universal solvent is accentuated, not just for its ubiquity on Earth but for its intrinsic chemical properties that facilitate both hydrolytic and biosynthetic catalytic reactions. The role of water becomes pivotal when considering the biochemical universality across disparate ecological niches. Lastly, the thesis underscores the continual energy influx as a quintessential element, a mechanism to maintain an internal entropy that is perpetually lower than the external environment, ensuring the thermodynamic viability of the living system.

These fundamental assumptions, while based on the observed parameters of terrestrial life, aim to provide a foundational framework for extrapolating the detection of life in

extraplanetary contexts. It is with the acknowledgment of these terrestrial biases that this research proceeds, with the hope that future explorations and discoveries will either validate or refine these foundational premises.

This thesis seeks to embark on an intensive study of the JSC Mars-1A Martian Regolith simulant and various clay simulants, exploring their properties and their interactions under Mars-analogous conditions. It also evaluates the applicability and effectiveness of current life detection methodologies when brought to bear on these simulants. In the pursuit of enhancing our capacity to detect life, the work encapsulated within this thesis presents the design, optimization, and implementation of novel methodologies for detecting biomarkers. These markers can potentially provide evidence of past life, precursors to future life, and even conditions inhibitive to life in soil simulants. Moreover, the study spearheads the development and application of tests geared towards supporting the detection of microbial life in soil samples. All this research pivots around the use of soil simulants and analogues to emulate specific properties of Martian regolith. Ultimately, the objective is to contribute substantially to the evolution of more robust and effective strategies for the search for life on Mars.

The ensuing chapters of this thesis primarily concentrate on the advancement of methodologies for the identification of specific biomolecular signatures indicative of life. Chapter two is focused on the formulation of simplified techniques for the quantification of proteins present in Martian soil simulants, employing Coomassie Brilliant Blue G-250 dye as the key reagent. Chapter three undertakes the task for the detection of nucleic acids and their respective constituents, deploying integrated fluorometric methods. This approach facilitates a more holistic comprehension of their significance in the genesis and sustenance of life. Turning the focus towards the evolution of metabolism, seen as a universally shared parameter in the emergence and development of life. Chapter four scrutinizes the search for metabolites that could potentially serve as distinct markers for life detection with a battery of biochemical methods and mass spectrometry. The final fifth chapter is predicated on the hypothesis that microbial life forms may exhibit enhanced resilience against the stringent

conditions of Mars or other extraterrestrial environments. Herein, methods for the investigation of the presence of these microbes are introduced. This novel approach may bolster our understanding and exploration of potential microbial extraterrestrial life.

This thesis predominantly focuses on exploring the potential for life on Mars; however, it is cognizant of the broader spectrum of extraterrestrial life search, extending beyond the red planet. Moons such as Enceladus and Europa, orbiting Saturn and Jupiter respectively, emerge as promising candidates for life detection, courtesy of the intriguing phenomena of ice plumes (McKay, 2008). These emissions, propelled from the moon's surface, are thought to emanate from underlying oceans, opening up possibilities of biotic systems veiled beneath their icy exteriors. In this context, while the investigative protocols and discoveries delineated herein are tailored to Martian terrains, their applicability and pertinence may transcend these boundaries. A constructive extrapolation of these methodologies could facilitate nuanced explorations into the biochemical potentials harbored by Enceladus and Europa, fostering a more comprehensive understanding of extraterrestrial biological occurrences.

Hence, this study, although rooted in Martian exploratory paradigms, serves as a constituent of the intricate tapestry of astrobiological research. The insights gleaned contribute substantively to a collective endeavour, amalgamating diverse planetary and lunar investigations, each integral in piecing together the enigmatic jigsaw of life's existence beyond our terrestrial frontiers.

1.2 References

ABREVAYA, X. C., MAUAS, P. J. & CORTON, E. 2010. Microbial fuel cells applied to the metabolically based detection of extraterrestrial life. *Astrobiology,* 10**,** 965-71.

ARVIDSON, R. E., SQUYRES, S. W., ANDERSON, R. C., BELL, J. F., BLANEY, D., BRÜCKNER, J., CABROL, N. A., CALVIN, W. M., CARR, M. H., CHRISTENSEN, P. R., CLARK, B. C., CRUMPLER, L., DES MARAIS, D. J., DE SOUZA, P. A., D'USTON, C., ECONOMOU, T., FARMER, J., FARRAND, W. H., FOLKNER, W., GOLOMBEK, M., GOREVAN, S., GRANT, J. A., GREELEY, R., GROTZINGER, J., GUINNESS, E., HAHN, B. C., HASKIN, L., HERKENHOFF, K. E., HUROWITZ, J. A., HVIID, S., JOHNSON, J. R., KLINGELHÖFER, G., KNOLL, A. H., LANDIS, G., LEFF, C., LEMMON, M., LI, R., MADSEN, M. B., MALIN, M. C., MCLENNAN, S. M., MCSWEEN, H. Y., MING, D. W., MOERSCH, J., MORRIS, R. V., PARKER, T., RICE, J. W., RICHTER, L., RIEDER, R., RODIONOV, D. S., SCHRÖDER, C., SIMS, M., SMITH, M., SMITH, P., SODERBLOM, L. A., SULLIVAN, R., THOMPSON, S. D., TOSCA, N. J., WANG, A., WÄNKE, H., WARD, J., WDOWIAK, T., WOLFF, M. & YEN, A. 2006. Overview of the Spirit Mars Exploration Rover Mission to Gusev Crater: Landing site to Backstay Rock in the Columbia Hills. *Journal of Geophysical Research: Planets,* 111**,** n/a-n/a.

AZUA-BUSTOS, A., FAIREN, A. G., SILVA, C. G., CARRIZO, D., FERNANDEZ-MARTINEZ, M. A., ARENAS-FAJARDO, C., FERNANDEZ-SAMPEDRO, M., GIL-LOZANO, C., SANCHEZ-GARCIA, L., ASCASO, C., WIERZCHOS, J. & RAMPE, E. B. 2020. Inhabited subsurface wet smectites in the hyperarid core of the Atacama Desert as an analog for the search for life on Mars. *Sci Rep*, 10, 19183.

BAK, E. N., ZAFIROV, K., MERRISON, J. P., JENSEN, S. J. K., NØRNBERG, P., GUNNLAUGSSON, H. P. & FINSTER, K. 2017. Production of reactive oxygen species from abraded silicates. Implications for the reactivity of the

Martian soil. *Earth and Planetary Science Letters*, 473, 113-121.

BANERDT, W. B. & SMREKAR, S. E. 2019. *InSight* [Online]. Available: https://nssdc.gsfc.nasa.gov/nmc/spacecraft/display.action?id=INSIGHT [Accessed 27 August 2019].

BOSAK, T., MOORE, K. R., GONG, J. & GROTZINGER, J. P. 2021. Searching for biosignatures in sedimentary rocks from early Earth and Mars. *Nature Reviews Earth & Environment,* 2**,** 490-506.

BROWN, D., WEBSTER, G., BORJA, R. & STILES, L. 2008. *Mars Phoenix Lander Finishes Successful Work on Red Planet* [Online]. Available: https://www.nasa.gov/mission_pages/phoenix/news/phoenix-20081110.html [Accessed 21 August 2019].

CANNON, K. M., BRITT, D. T., SMITH, T. M., FRITSCHE, R. F. & BATCHELDOR, D. 2019. Mars global simulant MGS-1: A Rocknest-based open standard for basaltic martian regolith simulants. *Icarus,* 317**,** 470-478.

CRISP, J. A., ADLER, M., MATIJEVIC, J. R., SQUYRES, S. W., ARVIDSON, R. E. & KASS, D. M. 2003. Mars Exploration Rover mission. *Journal of Geophysical Research: Planets,* 108.

CUADROS, J., MAVRIS, C., MICHALSKI, J. R., NIETO, J. M., BISHOP, J. L. & FIORE, S. 2019. Abundance and composition of kaolinite on Mars: Information from NIR spectra of rocks from acid-alteration environments, Riotinto, SE Spain. *Icarus*, 330, 30-41.

DEMING, L. D. & SEAGER, S. 2017. Illusion and reality in the atmospheres of exoplanets. *Journal of Geophysical Research: Planets,* 122**,** 53-75.

DES MARAIS, D. J., NUTH, J. A., 3RD, ALLAMANDOLA, L. J., BOSS, A. P., FARMER, J. D., HOEHLER, T. M., JAKOSKY, B. M., MEADOWS, V. S., POHORILLE, A., RUNNEGAR, B. & SPORMANN, A. M. 2008. The NASA Astrobiology Roadmap. *Astrobiology,* 8**,** 715-30.

ESA. 2007. *How to find an extrasolar planet* [Online]. Available: https://www.esa.int/Our_Activities/Space_Science/How_to_find_an_extrasolar_planet [Accessed 20 August 2019].

ESA. 2016. *EXOMARS MISSION (2020)* [Online]. Available: http://exploration.esa.int/mars/48088-mission-overview/ [Accessed 25 August 2019].

ESA. 2017. *EXOMARS TRACE GAS ORBITER AND SCHIAPARELLI MISSION (2016)* [Online]. Available: http://exploration.esa.int/mars/46124-mission-overview/ [Accessed 20 August 2019].

ESA. 2018. *Why go to Mars?* [Online]. Available: https://www.esa.int/Our_Activities/Human_and_Robotic_Exploration/Exploration/Why_go_to_Mars [Accessed 20 August 2019].

ESA. 2019a. *All instruments onboard Rosalind Franklin Rover* [Online]. Available: https://www.esa.int/Our_Activities/Human_and_Robotic_Exploration/Exploration/ExoMars/All_instruments_onboard_Rosalind_Franklin_rover [Accessed 25 September 2019].

ESA. 2019b. *ESA's Mars Rover has a name - Rosalind Franklin* [Online]. Available: https://www.esa.int/Our_Activities/Human_and_Robotic_Exploration/Exploration/ExoMars/ESA_s_Mars_rover_has_a_name_Rosalind_Franklin [Accessed 25 September 2019].

FAIRÉN, A. G. 2008. Finding of unusual soil on Mars could stem from tools used. *Nature,* 456**,** 870-870.

FOMINA, M. & SKOROCHOD, I. 2020. Microbial Interaction with Clay Minerals and Its Environmental and Biotechnological Implications. *Minerals,* 10.

GEORGIOU, C. D., MCKAY, C. P., QUINN, R. C., KALAITZOPOULOU, E., PAPADEA, P. & SKIPITARI, M. 2019. The Oxygen Release Instrument: Space Mission Reactive Oxygen Species Measurements for Habitability Characterization, Biosignature Preservation Potential Assessment, and Evaluation of Human Health Hazards. *Life (Basel)*, 9.

GOOD, A. & JOHNSON, A. 2019. *New Finds for Mars Rover, Seven Years After Landing* [Online]. Available: https://www.nasa.gov/feature/jpl/new-finds-for-mars-rover-seven-years-after-landing [Accessed 12 August 2019].

HAYS, L., ACHENBACH, L., BAILEY, J., BARNES, R., BAROSS, J., BERTKA, C.,

BOSTON, P., BOYD, E., CABLE, M., CHEN, I., CIESLA, F. & MARAIS, D. D. 2015. NASA ASTROBIOLOGY STRATEGY. Available: https://astrobiology.nasa.gov/uploads/filer_public/01/28/01283266-e401-4dcb-8e05-3918b21edb79/nasa_astrobiology_strategy_2015_151008.pdf.

HEGDE, S., PAULINO-LIMA, I. G., KENT, R., KALTENEGGER, L. & ROTHSCHILD, L. 2015. Surface biosignatures of exo-earths: remote detection of extraterrestrial life. *Proc Natl Acad Sci U S A,* 112**,** 3886-91.

HORNECK, G., WALTER, N., WESTALL, F., GRENFELL, J. L., MARTIN, W. F., GOMEZ, F., LEUKO, S., LEE, N., ONOFRI, S., TSIGANIS, K., SALADINO, R., PILAT-LOHINGER, E., PALOMBA, E., HARRISON, J., RULL, F., MULLER, C., STRAZZULLA, G., BRUCATO, J. R., RETTBERG, P. & CAPRIA, M. T. 2016. AstRoMap European Astrobiology Roadmap. *Astrobiology,* 16**,** 201-43.

HOWELL, E. 2018. *CubeSats: Tiny Payloads, Huge Benefits for Space Research* [Online]. Space.com. Available: https://www.space.com/34324-cubesats.html [Accessed 30 September 2019].

JONES, N. 2018. *NASA's MAVEN Selfie Marks Four Years in Orbit at Mars* [Online]. Available: https://www.nasa.gov/feature/goddard/2018/maven-selfie-marks-four-years-in-orbit-at-mars [Accessed 18 August 2019].

JONES, N. N. 2019. *NASA's MAVEN Uses Red Planet's Atmosphere to Change Orbit* [Online]. Available: https://www.nasa.gov/feature/goddard/2019/maven-uses-atmosphere-to-change-orbit [Accessed 19 August 2019].

KLOPROGGE, J. T. T. & HARTMAN, H. 2022. Clays and the Origin of Life: The Experiments. *Life (Basel),* 12.

LEVIN, G. V. 1972. Detection of metabolically Produced labeled gas: The Viking Mars Lander. *Icarus,* 16**,** 153-166.

LEVIN, G. V. 1997. The Viking Labeled Release experiment and life on Mars. *Instruments, Methods, and Missions for the Investigation of Extraterrestrial Microorganisms,* 3111.

LINGAM, M. & LOEB, A. 2019. Relative Likelihood of Success in the Search for

Primitive versus Intelligent Extraterrestrial Life. *Astrobiology,* 19**,** 28-39.

MASETTI, M. 2015. *Weather or not…* [Online]. NASA. Available: https://asd.gsfc.nasa.gov/blueshift/index.php/2015/01/29/exoplanet-weather/ [Accessed 30 September 2022].

MCKAY, C. P. 2008. An approach to searching for life on Mars, Europa, and Enceladus. *Strategies of Life Detection*, 49-54.

NASA. 2012. *Mars Science Laboratory Landing* [Online]. Available: https://mars.nasa.gov/internal_resources/823/ [Accessed 30 September 2019].

NASA. 2013. *Space Debris and Human Spacecraft* [Online]. Available: https://www.nasa.gov/mission_pages/station/news/orbital_debris.html [Accessed 27 September 2019].

NASA. 2018a. *CubeSats Overview* [Online]. Available: https://www.nasa.gov/mission_pages/cubesats/overview [Accessed 28 September 2019].

NASA. 2018b. *Viking Mission to Mars* [Online]. Available: https://nssdc.gsfc.nasa.gov/planetary/viking.html [Accessed 21 August 2019].

NASA. 2019a. *Mariner 9 NSSDCA/COSPAR ID: 1971-051A* [Online]. Available: https://nssdc.gsfc.nasa.gov/nmc/spacecraft/display.action?id=1971-051A [Accessed 21 August 2019].

NASA. 2019b. *Mars 3 NSSDCA/COSPAR ID: 1971-049A* [Online]. Available: https://nssdc.gsfc.nasa.gov/nmc/spacecraft/display.action?id=1971-049A [Accessed 21 August 2019].

NASA. 2019c. *Mars 2020 Mission Overview* [Online]. Available: https://mars.jpl.nasa.gov/mars2020/mission/overview/ [Accessed 25 August 2019].

NASA. 2019d. *NASA MARS Curiosity Rover Results* [Online]. Available: https://mars.nasa.gov/msl/mission/science/results/ [Accessed Aug. 12 2019].

NAZ, N., LIU, D., HARANDI, B. F. & KOUNAVES, S. P. 2022. Microbial Growth in Martian Soil Simulants Under Terrestrial Conditions: Guiding the Search for Life on Mars. *Astrobiology,* 22**,** 1210-1221.

ORAVEC, H. A., ASNANI, V. M., CREAGE, C. M. & MORELAND, S. J. 2021. Geotechnical review of existing Mars soil simulants for surface mobility. *Earth and Space 2021.*

PATTY, C. H. L., TEN KATE, I. L., BUMA, W. J., VAN SPANNING, R. J. M., STEINBACH, G., ARIESE, F. & SNIK, F. 2019. Circular Spectropolarimetric Sensing of Vegetation in the Field: Possibilities for the Remote Detection of Extraterrestrial Life. *Astrobiology,* 19**,** 1221-1229.

PETIT, S. 2006. Chapter 12.6 Fourier Transform Infrared Spectroscopy. *Handbook of Clay Science.*

QUINN, R. C., MARTUCCI, H. F. H., MILLER, S. R., BRYSON, C. E., GRUNTHANER, F. J. & GRUNTHANER, P. J. 2013. Perchlorate Radiolysis on Mars and the Origin of Martian Soil Reactivity. *Astrobiology,* 13, 515-520.

QURESHI, B. 2023. Proliferation and survival analysis of rhizosphere soil bacteria in a new Mars soil simulant under Mars conditions.

SAUNDERS, R. S., ARVIDSON, R. E., BADHWAR, G. D., BOYNTON, W. V., CHRISTENSEN, P. R., CUCINOTTA, F. A., FELDMAN, W. C., GIBBS, R. G., KLOSS JR, C., LANDANO, M. R., MASE, R. A., MCSMITH, G. W., MEYER, M. A., MITROFANOV, I. G., PACE, G. D., PLAUT, J. J., SIDNEY, W. P., SPENCER, D. A., THOMPSON, T. W. & ZEITLIN, C. J. 2004. 2001 Mars Odyssey Mission Summary. *Space Science Reviews,* 110**,** 1-36.

SCHUBOTZ, F., SEPHTON, M. A. & DERENNE, S. 2022. Biomarkers in Extreme Environments on Earth and the Search for Extraterrestrial Life in Our Solar System. *Elements,* 18**,** 100-106.

SCHUERGER, A. C. & CLARK, B. C. 2007. Viking Biology Experiments: Lessons Learned and the Role of Ecology in Future Mars Life-Detection Experiments. *Space Science Reviews,* 135**,** 233-243.

SCHULZE-MAKUCH, D., HEAD, J. N., HOUTKOOPER, J. M., KNOBLAUCH, M., FURFARO, R., FINK, W., FAIRÉN, A. G., VALI, H., KELLY SEARS, S., DALY, M., DEAMER, D., SCHMIDT, H., HAWKINS, A. R., SUN, H. J., LIM, D. S. S., DOHM, J., IRWIN, L. N., DAVILA, A. F., MENDEZ, A. &

ANDERSEN, D. 2012. The Biological Oxidant and Life Detection (BOLD) mission: A proposal for a mission to Mars. *Planetary and Space Science,* 67**,** 57-69.

SNELLEN, I. A. G., DE KOK, R. J., LE POOLE, R., BROGI, M. & BIRKBY, J. 2013. Finding Extraterrestrial Life Using Ground-Based High-Dispersion Spectroscopy. *The Astrophysical Journal,* 764.

SQUYRES, S. W., ARVIDSON, R. E., BOLLEN, D., BELL, J. F., BRÜCKNER, J., CABROL, N. A., CALVIN, W. M., CARR, M. H., CHRISTENSEN, P. R., CLARK, B. C., CRUMPLER, L., DES MARAIS, D. J., D'USTON, C., ECONOMOU, T., FARMER, J., FARRAND, W. H., FOLKNER, W., GELLERT, R., GLOTCH, T. D., GOLOMBEK, M., GOREVAN, S., GRANT, J. A., GREELEY, R., GROTZINGER, J., HERKENHOFF, K. E., HVIID, S., JOHNSON, J. R., KLINGELHÖFER, G., KNOLL, A. H., LANDIS, G., LEMMON, M., LI, R., MADSEN, M. B., MALIN, M. C., MCLENNAN, S. M., MCSWEEN, H. Y., MING, D. W., MOERSCH, J., MORRIS, R. V., PARKER, T., RICE, J. W., RICHTER, L., RIEDER, R., SCHRÖDER, C., SIMS, M., SMITH, M., SMITH, P., SODERBLOM, L. A., SULLIVAN, R., TOSCA, N. J., WÄNKE, H., WDOWIAK, T., WOLFF, M. & YEN, A. 2006. Overview of the Opportunity Mars Exploration Rover Mission to Meridiani Planum: Eagle Crater to Purgatory Ripple. *Journal of Geophysical Research: Planets,* 111**,** n/a-n/a.

THEPLANETARYSOCIETY. 2019a. *Astrometry: The Past and Future of Planet Hunting* [Online]. Available: http://www.planetary.org/explore/space-topics/exoplanets/astrometry.html [Accessed 20 August 2019].

THEPLANETARYSOCIETY. 2019b. *Radial Velocity: The First Method that Worked* [Online]. Available: http://www.planetary.org/explore/space-topics/exoplanets/radial-velocity.html [Accessed 20 August 2019].

TU, V. M., RAMPE, E. B., BRISTOW, T. F., THORPE, M. T., CLARK, J. V., CASTLE, N., FRAEMAN, A. A., EDGAR, L. A., MCADAM, A., BEDFORD, C., ACHILLES, C. N., BLAKE, D., CHIPERA, S. J., CRAIG, P. I., DES MARAIS,

D. J., DOWNS, G. W., DOWNS, R. T., FOX, V., GROTZINGER, J. P., HAZEN, R. M., MING, D. W., MORRIS, R. V., MORRISON, S. M., PAVRI, B., EIGENBRODE, J., PERETYAZHKO, T. S., SARRAZIN, P. C., SUTTER, B., TREIMAN, A. H., VANIMAN, D. T., VASAVADA, A. R., YEN, A. S. & BRIDGES, J. C. 2021. A Review of the Phyllosilicates in Gale Crater as Detected by the CheMin Instrument on the Mars Science Laboratory, Curiosity Rover. *Minerals,* 11.

VOOSEN, P. 2019. *Cost of Mars 2020 mission may rise by up to 15%* [Online]. Science and AAAS. Available: https://www.sciencemag.org/news/2019/03/cost-mars-2020-mission-may-rise-15 [Accessed 30 September 2019].

WILLIAMS, D. R. 2014. *Mars Pathfinder Project Information* [Online]. Available: https://nssdc.gsfc.nasa.gov/planetary/mesur.html [Accessed 21 August 2019].

ZENG, X., LI, X., WANG, S., LI, S., SPRING, N., TANG, H., LI, Y. & FENG, J. 2015. JMSS-1: a new Martian soil simulant. *Earth, Planets and Space,* 67.

Chapter 2: Development of a simple step and ultrasensitive protein assays for planetary exploration

Abstract

Accurate and efficient detection of proteins in extra-terrestrial environments represents a pivotal element in the search for life beyond Earth. This chapter elucidates the meticulous optimization of a Bradford-based assay designed for protein quantification in simulated Martian soil. A series of experimental investigations were conducted to optimize various parameters, including protein spiking volume, extraction time, and the concentration of the spiking protein. Methanol emerged as an effective solvent for spiking, and a speed vacuum concentrator was identified as the preferred means of evaporating the spiking solution. Statistical analyses confirmed that the method is robust across a range of conditions, obviating any significant difference among varying spiking volumes, protein concentrations, or extraction times. Additionally, the optimized assay was validated using bacterial proteins extracted from E. coli cultures, further substantiating its applicability for microbial life detection. Limitations concerning the assay's efficacy in clay substrates were noted, opening avenues for future research. This optimized protocol represents a significant contribution to astrobiological research, presenting an economical, streamlined, and sensitive approach for protein detection with potential for application in future Martian missions.

2.1 Introduction

2.1.1 Protein Detection for exoplanet missions

The quest for extra-terrestrial life is fundamentally anchored in the identification of biosignatures—distinctive physical and chemical markers that serve as evidence of biological activity. These indicators not only illuminate the essential prerequisites for the emergence of life but also constitute the cornerstone of current endeavours in exoplanetary exploration. It is widely accepted within the scientific community that the basic building blocks for life, as we understand it, involve the presence of liquid water as well as organic molecules—complex structures primarily composed of carbon and hydrogen atoms in association with elements such as oxygen, nitrogen, and sulfur (Bains, 2004). While it remains an open question whether life forms beyond Earth would share the same biochemical foundation as terrestrial organisms, utilizing Earth-based macromolecules provides an indispensable framework for detecting potential biosignatures in extra-terrestrial environments.

One such category of these macromolecules is proteins, the integral constituents of biological systems that drive essential cellular processes such as analysis, metabolism, and signalling. With proteins comprising nearly 55% of the dry weight of bacterial species such as E. coli, they represent the most abundant biomolecule in terms of mass (Milo and Phillips, 2015). This widespread prevalence implies that detection and analysis of proteins in extra-terrestrial environments could serve as a dependable signifier of biological activity beyond our home planet.

Furthermore, the study of proteins in astrobiology transcends the simple quest for life detection. Proteins, with their extensive array of structures and functions, can yield crucial insights into the metabolic activities and environmental adaptability of potential extra-terrestrial life forms (Tomanek, 2014, Schwieterman et al., 2018). Such knowledge can subsequently refine our comprehension of the boundaries of life and its potential distribution across the universe. For instance, the Martian meteorite Allan Hills (ALH) 84001, founded on the Antarctic ice in 1984, was proposed to contain

biosignatures, biogenic minerals, and microfossils (McKay et al., 1996). Although it was indicated that most of the organic carbon and amino acids in ALH 84001 is terrestrial (Bada et al., 1998, Jull et al., 1998), this work has promoted the development of Mars exploration program focused on the search for life.

Given their universal nature and shared characteristics across all known life forms, proteins provide an optimal framework for searching for life across diverse environments. Hence, proteins emerge as an excellent candidate category of macromolecules for detecting life signatures in potential habitable regions on the soils of celestial bodies under investigation.

2.1.2 Existing protein detection methods and their limitations

The detection of extra-terrestrial life is increasingly facilitated by advanced space-based observational technologies, particularly sophisticated telescopes. These instruments enable the identification of atmospheric biosignature gases, which are volatile compounds produced by biological processes that can accumulate to levels that are discernible in the atmosphere of an exoplanet (Seager, 2014). Notably, NASA's Spitzer Space Telescope has reported the presence of precursor molecules essential for protein formation within the habitable zone surrounding a distant star (NASA, 2005). As a result, proteins are emerging as significant biosignatures in the ongoing search for extra-terrestrial life.

The most commonly used methods to quantify proteins are the Kjeldahl method, the Biuret methods (i.e. the BCA assay and the Lowry assay), the colorimetric (dye-based) methods (i.e. the Bradford assay) and the fluorescent dye methods (i.e. the EZQ fluorescent assay and the qubit protein assay). The Kjeldahl method is the classical method to analyse and quantify the nitrogen content in organic compounds. It is a reliable and well-established technique to measure total nitrogen content in a wide range of samples which is relatively simple and straightforward, with a low cost of equipment and reagents (Aguirre, 2023). Due to the nitrogen content of a protein is relatively constant (usually accounts for about 16%), the protein content can be

calculated from its nitrogen content, which is the protein content = nitrogen content / 16% (Kimberly and Roberts, 1905). So, this method is also the classical protein quantification method. However, the Kjeldahl method requires the use of concentrated sulfuric acid, which may not be practical or safe to use in the harsh Martian environment. The method also involves several steps, including digestion and titration, which may be difficult to perform in a low-gravity or high-radiation environment (Aguirre, 2023).

The Biuret methods is based on that polypeptides can coordinate with copper to form purple protein-copper chelate under alkaline conditions which can be detected at 540 nm (UV range) (John Savory et al., 1968). The Lowry assay has the same colour reaction principle with the Biuret methods but has the secondary reagent, Folin-phenol reagent, to improve the sensitivity (Lowry et al., 1951). Such an approach is very prone to copper ions interferences from the soil matrix. Additionally, quantification of organic N in soil samples could be an approach to express protein content, however, the common methods require a significant amount of incubation time and are also prone to inorganic nitrogen (i.e. nitrate and ammonium) (Hurisso et al., 2018).

The Bradford assay is another colorimetric dye-based method, which is the widely used to detect proteins. It is based on that Coomassie Brilliant Blue (CBB) G-250 dye which can combine with proteins in an acidic solution and the color of the generation of absorbing complexes (Compton and Jones, 1985). The hydrophobic nature and molecular attraction in the binding of the dye to proteins were extensively describe by Georgiou et al. (2008), and the method was later revised in a quick and easy to perform assay with high sensitivity (Grintzalis et al., 2015).

The methods using fluorescent dyes rely on the generation of complexes when the reagents combine with proteins and directly detect the increase in fluorescence associated with the bound dye. These assays have excellent sensitivity and can be adapted for automated handling in high-throughput applications (Hawe et al., 2008). For identifying proteins, it is commonly used Liquid chromatography–mass spectrometry (LC-MS).

Among these methods for quantifying proteins, the colorimetric dye-based methods, such as the Bradford assay, are preferred than others as the operation of Kjeldahl method is complicated and time-consuming operation, and copper and some common reducing agents such as dithiothreitol (DTT) could be interfering in the Biuret methods. As for the fluorescent dye methods, although they have many advantages, they require specialized equipment which is a big departure from the aim to develop a simple assay. The flexibility and accuracy of Bradford assay make it a widely used tool with diverse applications in various fields such as geosciences, agriculture, biotechnology, and medical research. In geosciences, the Bradford assay can be used to quantify the protein content of the soil, a critical factor in understanding nutrient cycling and biological activity in various soil types (Redmile-Gordon et al., 2013). This information is valuable in improving soil management and agricultural practices. Similarly, in agriculture, the Bradford assay can be utilized to monitor the quality and quantity of protein in animal feed, an important aspect of ensuring animal growth and health (Nielsen, 2021). It can also be used to determine the protein content of plant-based foods, essential for human nutrition (Kumar et al., 2019). In the context of astrobiology, it holds the potential to detect and quantify proteins in extra-terrestrial environments, opening new possibilities for exploring the potential for life beyond Earth.

2.1.3 Methods for identifying proteins

There are two main analytical strategies commonly used in the proteomics to separate and identify proteins: the “top-down” and the “bottom-up” approaches (Wu et al., 2012). In the “top-down” approach, it is commonly used the liquid chromatography–mass spectrometry (LC-MS). The analytical methodology under consideration integrates liquid chromatography (LC) or its high-performance variant (HPLC) for the fractionation of proteins, with mass spectrometry (MS) for their subsequent identification. This synergistic approach capitalizes on the high molecular specificity of MS and the sensitivity of chromatographic separation, offering a powerful toolset for comprehensive protein analysis. (Wu et al., 2012, Domon and Aebersold, 2006). Another approach, which was proposed by O'Farrell (1975) is two-dimensional gel

electrophoresis (2-DE). This method can be used to detect and analyze proteins from complex biological sources. It is based on the different isoelectric point of proteins to separate in the first dimension and the different molecular weight with sodium dodecyl sulfate electrophoresis in the second dimension. Due to the different separation principle, the distribution of protein spots is homogeneous across a two-dimensional gel (O'Farrell, 1975).

The “bottom-up” approach can be considered as supplementary of the “top-down” approach. This is firstly digested proteins into peptides and then using chromatography to separate and mass spectrometry to identify (Washburn et al., 2001, Wu et al., 2012). Although it is more difficult to separate and identify loads of peptides at the same time compared with intact proteins, due to the higher sensitivity to peptide level of chromatography, this approach can characterize more proteins.

2.1.4 Soil proteomics

The complexity of proteins results in the richness of information extractable from soil but there are not many techniques for analysing this information. Regarding this situation, soil proteomics was introduced at the molecular level for analysing proteins in soil. Generally, the methods for soil proteomics include extraction methods and analytical methods. The aim to analytical methods determined the selection of extraction methods. For example, if the aim to analytical methods was to evaluate the function of protein, including enzyme activity, then the extraction methods needed to protect the function as much as possible. Conversely, if the aim to analytical methods was to compare the molecular size or for the immunological detection, the extraction methods could be less conservative (Ogunseitan, 2006).

Analysing proteins from the extracts of soil can be interfered by the substances originally from the soil (Gillespie et al., 2011). Due to the existence of substantial interfering substances in soil, it is challenging to get enough high-quality protein samples for analysing in mass spectrometry, as well as 2-DE-based proteomic analysis (Chen et al., 2009). For example, one of the interfering substances is polyphenols,

which can interfere the quantification of extracted protein. In the research of Wright and Upadhyaya (1996), it presented a method using citrate to extract soil which can minimize this interference. In colorimetric assays, the accuracy of quantifying proteins can be affected by not only chemical interference such as polyphenols, but also physical interferences, such as scattering, and physicochemical effects (i.e. the sorption of suspended soil samples) (Redmile-Gordon et al., 2013). Thus, how to minimize the influence of these interferences is the key factor to optimize colorimetric assays for quantifying proteins.

Besides colorimetric assays, polyacrylamide gel electrophoresis (PAGE) is another approach widely used for analysing proteins in the context of soil proteomics. 2,2,2-Trichloroethanol (TCE) can provide the possibility to fluorescent visible detection of proteins by adding into polyacrylamide gels before polymerization (Ladner et al., 2004). This method is based on the tryptophan in proteins can react with trihalocompounds to produce fluorescence and the protein bands can be detected by a transilluminator. It omits the staining step and can also observe integral membrane proteins which cannot be dyed by CBB (Patton, 2000, Ladner et al., 2004). Another common method used in polyacrylamide gel is the silver staining. After finishing the electrophoretic separation on polyacrylamide gels, the silver ions in the silver staining can combine with proteins to form a visible image (Chevallet et al., 2006). This assay is considered as an economical and simple way with high sensitivity to detect proteins, and it is also provide the compatibility to the further analysing process, such as mass spectrometry analysis (Chevallet et al., 2006).

2.1.5 Structure of the chapter

This chapter primarily aimed to optimise a Bradford-based assay that is both economical and straightforward for the precise measurement of proteins extracted from Martian soil simulants. The overarching objective was to evaluate the suitability of this method for prospective planetary exploration missions. Successfully implementing this technique could substantially enhance the capability to identify potential protein biosignatures on Mars and other celestial bodies, thereby aiding towards the

comprehension of genesis and evolution of life across the cosmos.

This chapter elaborated on the process of optimizing the Bradford-based assay, detailing the specific steps undertaken to improve its sensitivity and reproducibility. Considering the expected conditions on the Martian surface, this study has considered the impacts of ultraviolet (UV) radiation and low temperatures as potential challenges for the preservation of protein signatures. The guiding hypothesis is that the refined assay would exhibit high sensitivity and reproducibility. These characteristics would facilitate the detection of protein signatures potentially preserved on the Martian surface, despite the severe impact of UV radiation exposure.

2.2 Materials and methods

2.2.1 Protein quantification by Coomassie Brilliant Blue

The assay is based on the electrostatic reaction of proteins with the Coomassie Brilliant Blue (CBB) G-250 dye in a rapid microplate method under acidic conditions (Grintzalis et al., 2015). The CBB solution (60 mg CBB in 100 ml 2M HCl, filtered to remove undissolved dye particulates) was diluted 2-fold with 2M HCl prior to use (designated as CBB:2M HCl) and a series of bovine serum albumin (BSA) standards (2-20 µg/ml) were prepared in ddH_2O. The samples and standards were assayed for protein concentration according to **Table 2.1**.

Table 2.1. Bradford assay conditions. The volumes are in µl. RB: Reagent Blank, S: Sample or Standard.

Reagents	**RB**	**S**
Sample appropriately diluted in ddH_2O or BSA standard in ddH_2O	-	200
ddH_2O	200	-
CBB:2M HCl	50	50

The mixtures were incubated for 10 minutes at room temperature and the absorbance was measured at 610 nm. The net absorbance (derived from the absorbance difference of Sample minus Reagent Blank) was converted to protein concentration equivalents using the corresponding standard curve.

2.2.2 Spiking of soil samples with proteins

Soil simulants (50 mg JSC Mars-1A Martian Regolith Simulant) were incubated with 100 µl different protein solutions in methanol and then dried using the speed vacuum concentrator. Proteins were hypothesized to be adsorbed onto the JSC Mars-1A simulant. Thereafter, the proteins were extracted in ddH_2O and subjected to quantification through the Bradford assay. (**Figure 2.1**). To ensure the accuracy, control groups were made without soil as well as without the addition of any protein solution. The overall spiking process was optimized and will be described in the Results section.

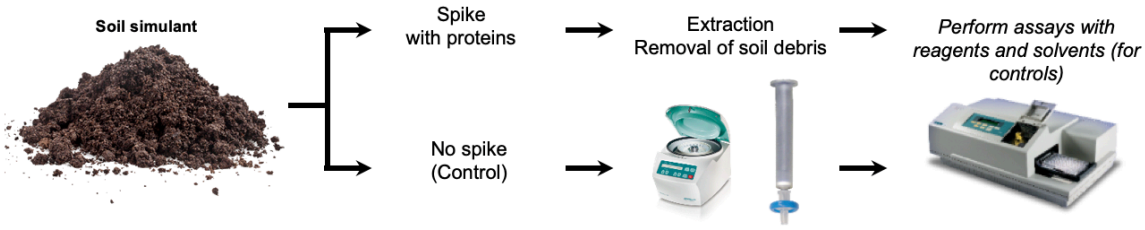


Figure 2.1. Spiking methods of soil samples with proteins.

2.2.3 Spiking of soil samples with proteins under UV exposure

Soil (50 mg JSC Mars-1A Martian Regolith Simulant) was incubated with 100 µl different protein solutions in methanol and then dried using the speed vacuum concentrator. Proteins were suspected remain into soils and then spiked soils were exposed under UV radiation at the UV dosage of 72 kJ/hm^2 (Carrier et al., 2019). Following, they were extracted in ddH_2O and quantified by the Bradford assay (**Figure 2.2**). To ensure the accuracy, control groups were made without UV exposure spiked soils.

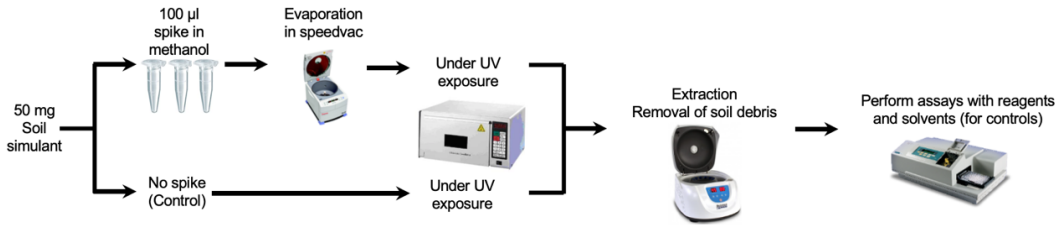


Figure 2.2. Spiking methods of soil samples with proteins under UV exposure.

2.2.4 Bacteria culture media preparation

Luria Bertani (LB) broth media was prepared by dissolving 20 g of LB in 1 litre of ddH_2O. The broth was transferred to Duran bottles and sterilised by autoclaving for 15 minutes at 121˚C. Sterile LB broth media was stored at 4˚C until use to prevent any contamination.

2.2.5 Escherichia coli culturing and soil spiking

Escherichia coli (E. coli) was cultured at 30°C under agitation at 200 rpm. From a liquid culture in LB broth media, a bacterial suspension was prepared in Eppendorf tubes and centrifuged (to clear media) at 6,000 *g* for 5 minutes at room temperature. The supernatant was removed, and the pelleted cells were re-suspended in ddH_2O. Cells were precipitated again by centrifugation at 6,000 *g* for 5 minutes and the supernatant was discarded. For generating a protein homogenate, the pelleted cells were re-suspended in 100 mM NaOH and homogenised by heating at 95℃ in a water bath for 2 hours. The protein homogenate was cleared from cellular debris by centrifugation at 6,000 g at room temperature for 5 minutes and the clear protein supernatant was collected and assayed for protein (by the Bradford assay) and used for spiking.

2.3 Results

2.3.1. Development and optimisation of the protein quantification assay

To troubleshoot the protein detection method, initially, the acidity of the CBB assay reaction was determined from 0 to 3 M HCl **(Figure 2.3)**. For the Bradford assay, the concentration to protons is crucial and a sufficient concentration is required for the reaction, however, extensive acidity resulted in interference and decrease of the sensitivity of the method.

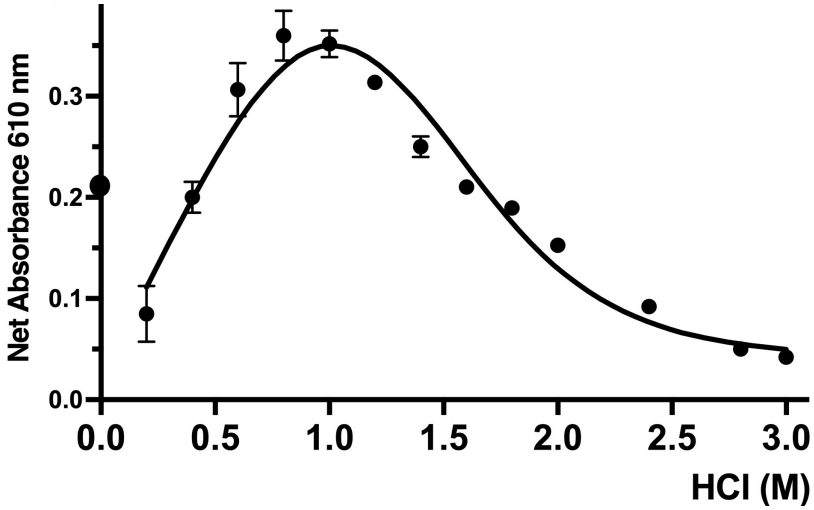


Figure 2.3. Optimization of the acidity of the protein assay. The impact of acidity to the reaction was observed with BSA (20 µg BSA/ml) mixed with 25 µl CBB and different concentrations of HCl (M).

Additionally, the time of incubation of the reaction was also explored with a series of protein standards, and the reaction signal remained stable from five up to at least thirty minutes, which allowed a significant time window for the feasibility of the method and a fast test **(Figure 2.4)**.

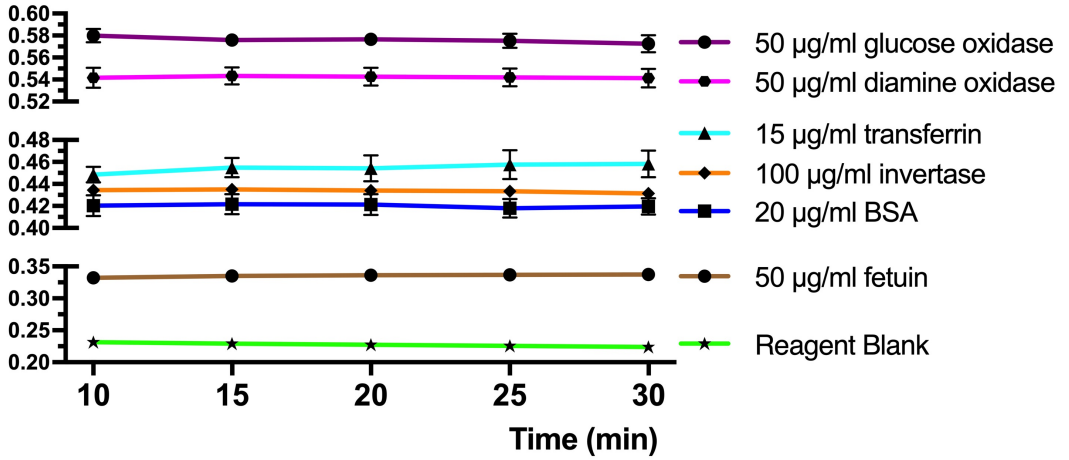


Figure 2.4. Optimization of the signal stability over time. Data represent average±SD (N=3).

Subsequent to the successful optimization of the assay parameters, the performance characteristics of the refined method were evaluated. A critical aspect of this evaluation involved generating standard curves for different proteins under the established optimal conditions **(Figure 2.5)**. These standard curves served as critical tools for assessing the assay's sensitivity and specificity, as well as for enabling precise quantification of proteins in the spiked Martian soil simulants.

Additionally, a comprehensive examination of the protein detection limits and linear range of the CBB assay was conducted. The findings from this assessment, as delineated in **Table 2.2** provided significant insights into the dynamic range and lower detection limits of the optimised assay. This information is indispensable for understanding the assay's ability to accurately and reliably detect low concentrations of proteins, a feature that is of paramount importance in the search for potential biosignatures in planetary exploration missions.

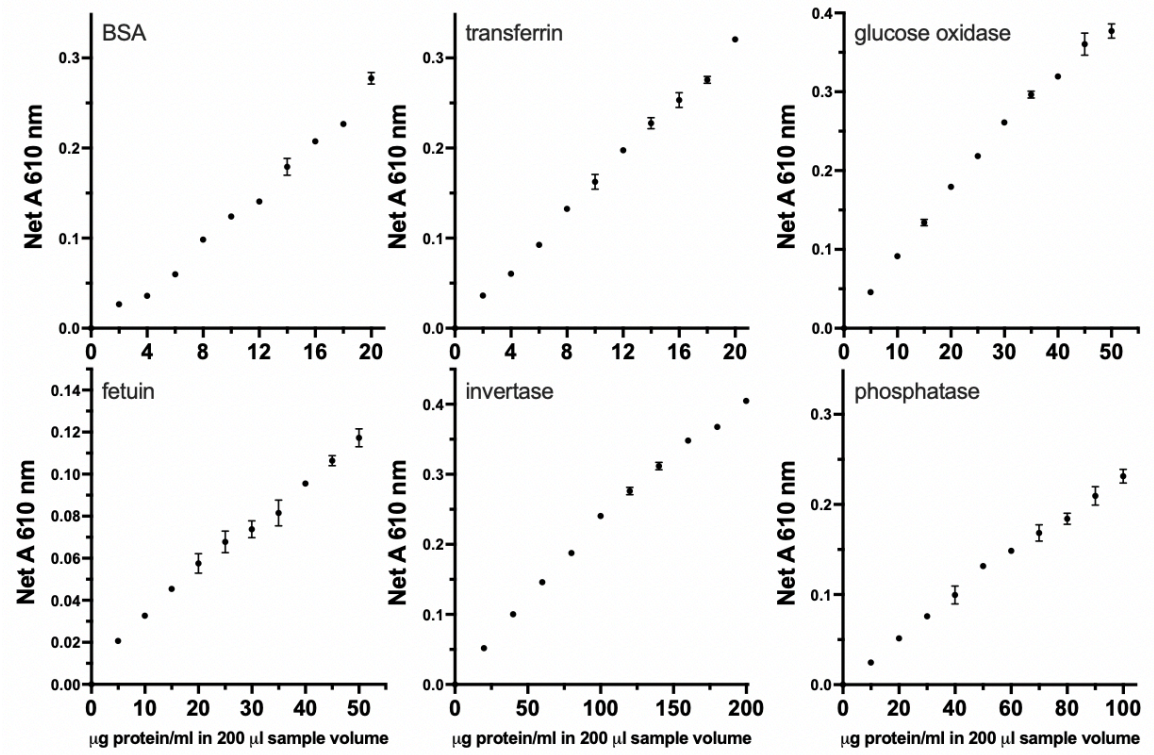


Figure 2.5. Linear standard curve for different proteins in ddH2O. Data represent average±SD (N=3).

Table 1. Protein detection limits and linear range for CBB assay.		
Protein	**Linear standard curve (μg protein/ml in 200 μl sample volume)**	**Standard curve slope**
BSA	2-20	0.0128
Transferrin	2-20	0.0159
Alkaline phosphatase	10-100	0.0024
Fetuin	5-50	0.0024
Diamine oxidase	5-50	0.0071
Glucose oxidase	5-50	0.0081
Invertase	20-200	0.0022
Myoglobin	3-30	0.0095
Alginate lyase	10-100	0.0024
Proteinase K	5-50	0.0065
Trypsin	40-400	0.0011
Catalase	5-50	0.0085
Hemoglobin	3-30	0.0148
Lysozyme	5-50	0.003
Lipase	100-1000	0.0001

Following, different spiking approaches were explored in relevance to the spiking volume, the amount of soil spiked, the concentration of spiking protein, the solvent used for spiking, the method of evaporation (on soil) and the protein standard used. First, two options of spiking solvent (methanol and water) were explored for spiking protein standards of BSA. BSA standards (400 µg/ml) prepared in methanol and water were spiked equally on soil and evaporated using a speed vacuum concentrator (**Figure 2.6**). The initial observation of note is the statistically robust divergence in the performance of methanol and water as a solvent for protein spiking. Specifically, methanol demonstrated a superior capability for achieving elevated levels of protein absorption, thereby suggesting its preferential utility in the protein spiking process.

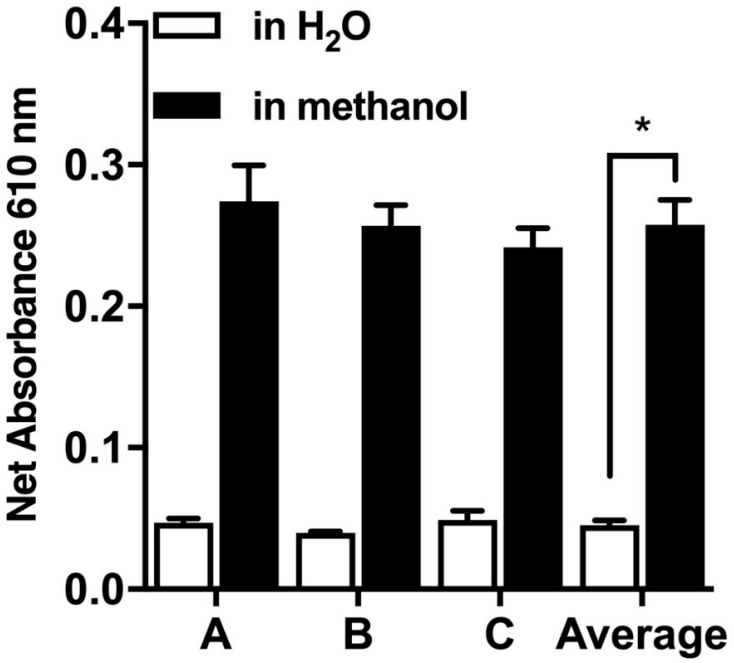


Figure 2.6. Exploring the difference in spiking solvent for spiking proteins. Data represent average±SD, for three independent replicates of each standard (A, B and C) and three (N=3) technical replicates. *Statistically significant by Student's *t*-test.

Following the initial experiments, the drying method was explored comparing oven-drying methods with speed vacuum concentrator (Speedvac) (**Figure 2.7**). An aliquot of 500 µg/ml Bovine Serum Albumin (BSA) dissolved in methanol was spiked to 50 mg of soil simulants in Eppendorf tubes. These were then dried either by different temperature levels in an oven or using a speed vacuum concentrator. The rationale

behind exploring various oven temperatures was to determine their influence on spiking efficiency.

Subsequently, the spiked soil simulants were evaluated using a biochemical technique (**Figure 2.7A**). This evaluation affirmed the hypothesis that temperature can indeed instigate a change in spiking efficiency. A statistical comparison between oven temperatures from 20 ºC to 37 ºC and the use of a speed vacuum concentrator revealed no significant difference. However, a divergence arose when the oven temperature exceeded 44 ºC, where a statistically significant difference was observed. Furthermore, this difference became progressively more pronounced with increasing temperatures, suggesting that higher temperatures could detrimentally impact the spiking efficiency.

After determining the optimum temperature in oven (37 ºC), the drying method was explored for alternatives using well plates in oven and then compared with Eppendorf tubes in oven and speed vacuum evaporator. 500 µg/ml BSA dissolved in methanol was spiked on soil in well plates (dried in the oven at 37 ºC) or Eppendorf tubes (dried in either the oven or speed vacuum concentrator) and protein was assessed by the Bradford assay (**Figure 2.7B**). The result indicated that there was no significant difference among using different well plates to spike soil in oven compared with spiking in speed vacuum evaporator. It provided flexibility in the choice of drying techniques, which could have implications for the efficiency, cost, and ease of the protein detection process during planetary missions. It might also allow researchers to choose the method most suitable for their specific experimental setup and constraints.

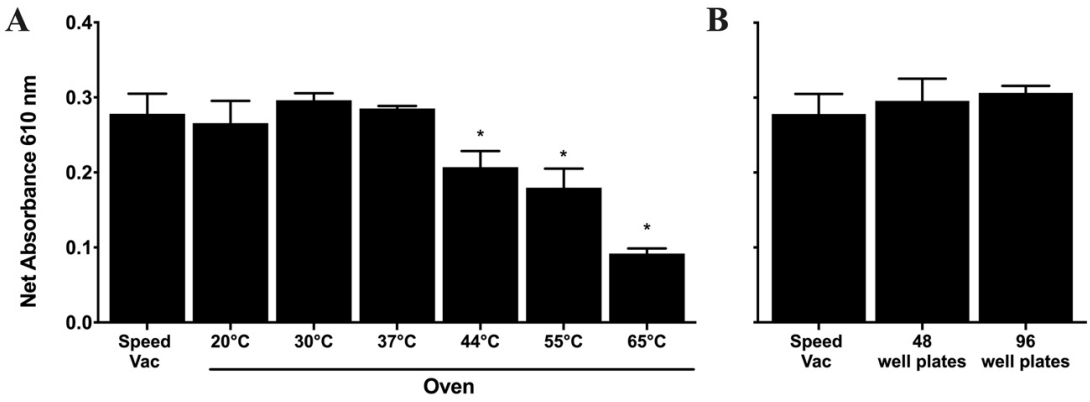


Figure 2.7. Optimization of the spiking method. A. BSA was spiked in methanol under different drying options and was recovered following extraction in 1 ml ddH_2O. B. Spiking soils in oven using well plates and speed vacuum evaporator in Eppendorf tubes. Data represent average±SD (N=3) and were considered statistically significant by Student's t-test compared to the speedvac drying method.

Upon the successful optimisation of the protein spiking process, the subsequent step involved examining the efficiency of protein extraction, with particular attention paid to both the volume and duration of extraction (**Figure 2.8**). It was observed that minimal volumes, less than 1 ml of deionised water (ddH_2O) were sufficiently competent for the consistent extraction of spiked protein from soil samples. This outcome facilitated the miniaturisation of the established method (**Figure 2.8A**). Additionally, the duration of extraction was scrutinised, and it was determined that vortexing as brief as 10 minutes were adequate for the complete recovery of the protein (**Figure 2.8B**). Thus, these findings suggest that the extraction process can be conducted with smaller volumes and reduced durations, optimising the efficiency and practicality of the method for potential applications in planetary missions.

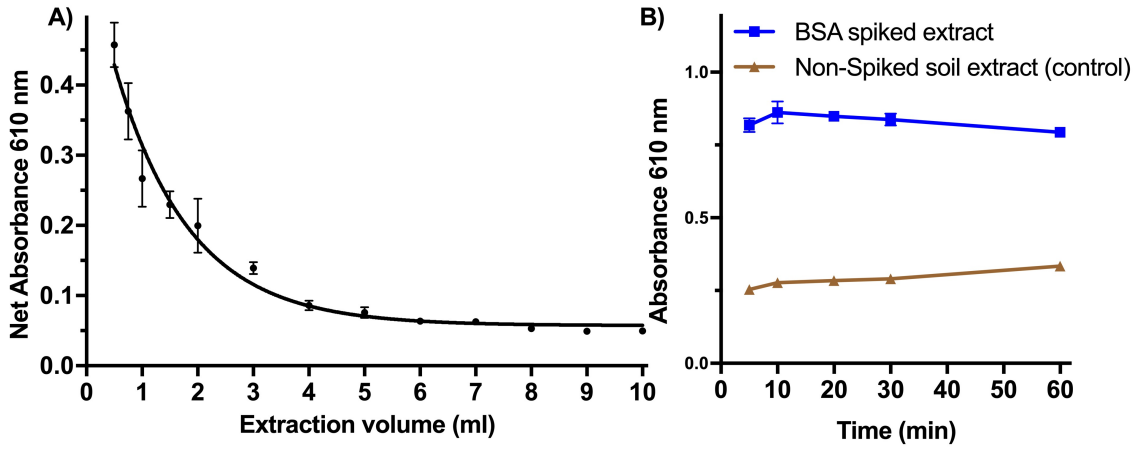


Figure 2.8. Optimization of the extraction step. (A) The volume (ml) of ddH_2O was tested for the extraction of spiked BSA (1 µg BSA/mg soil). (B) The time of extraction for 1 ml ddH_2O was explored for the recovery the spiked BSA (1 µg BSA/mg soil). Data represent average±SD (N=3).

The exploration of the spiking volume and concentration was undertaken with the stipulation of maintaining a steady final concentration of spiked soil (0.8 µg BSA/mg soil). The objective was to assess the influence of diverse spiking approaches on the final protein recovery (**Figure 2.9**). Under these varying conditions, an increase in spiking volume and soil quantity while maintaining a constant spiking protein concentration; a constant or increment in the soil quantity while the spiking protein concentration and spiking volume remained stable; or a constant or escalation in the spiking volume and the spiking protein concentration with the soil amount held constant - no significant deviation was observed among the distinct spiking strategies. These findings suggest a robustness to the spiking process, where different combinations of volume and concentration parameters can be employed without significantly impacting the final protein recovery. This flexibility could have valuable implications for tailoring the method to different contexts and sample sizes in future astrobiological applications.

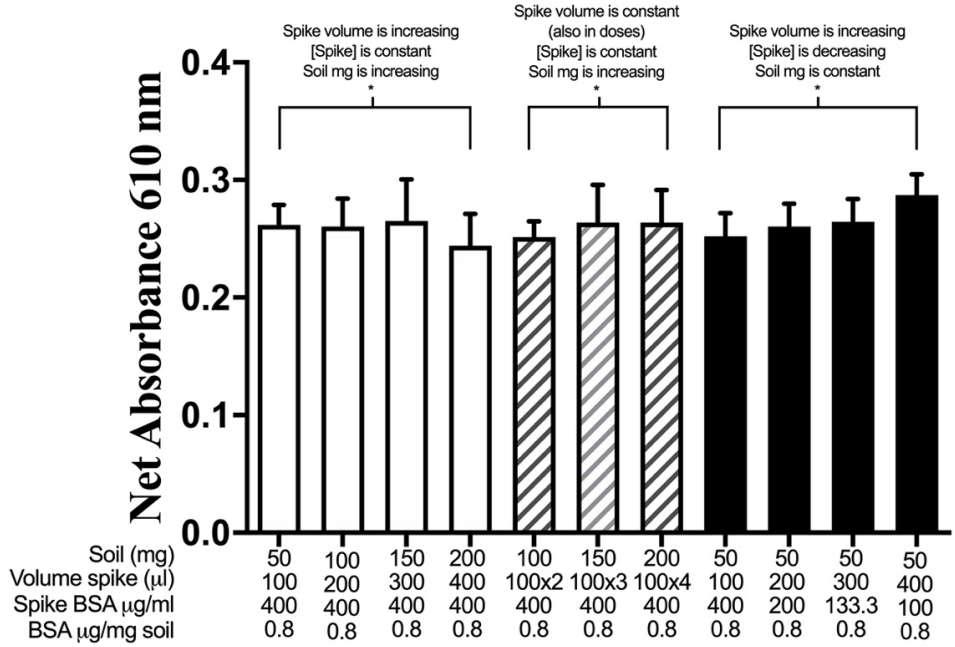


Figure 2.9. Impact of different spiking ways on the Bradford assay spiked soil. Data represent average±SD (N=3).

The subsequent investigation focused on the recovery of proteins from the BSA-spiked soil simulants. BSA-spiked soil simulants were extracted in water, with the resultant data then contrasted with equivalent concentrations of a pure BSA solution (**Figure 2.10**). 20 µg BSA (50µl from a 400 µg/ml BSA stock in methanol) was spiked in 50 mg soil simulants and extracted in 1 ml water (in total). The recovery of BSA from the soil simulants varied between 83.4% and 89.6% over four extraction replicates **(Figure 2.10A)**. The highest recovery was achieved by performing the extraction twice with 0.5 ml water each time. An increase in extraction repetitions led to an elevated absorbance of the control group, thereby diminishing the net absorbance of BSA. To validate the precision of this method, recovery was assessed at two distinct BSA concentrations **(Figure 2.10B)**. Amounts of 20 and 40 µg BSA were individually spiked onto 50 mg of soil simulants and subsequently extracted in 1ml of water. Recoveries were found to be 83.1% and 85.3%, respectively, with no significant difference identified. Increasing the extraction volume to 2ml for the 40 µg BSA spike, thereby creating equivalence to the 20 µg/ml BSA, resulted in a recovery of 86.8%. In summary, the data suggested that the average recovery of BSA from spiked soil simulants hovered around 86%,

indicating a reasonable degree of reliability in the protein extraction method from these complex matrices.

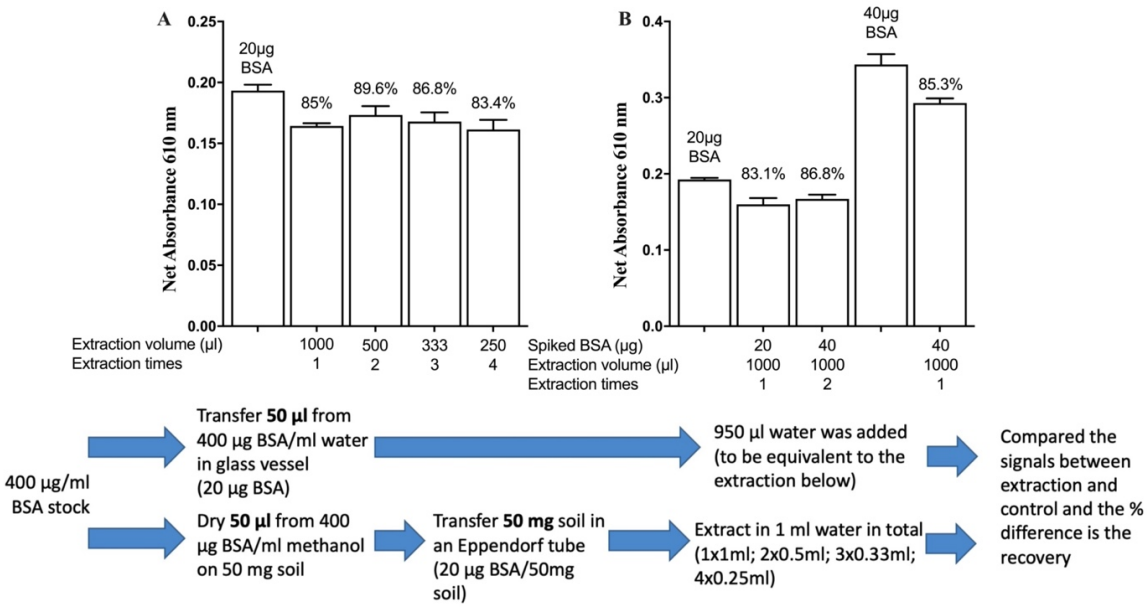


Figure 2.10. The recovery of proteins from spiked soil simulants. Data represent average±SD (N=3).

Having optimised all the parameters regarding the spiking approaches, the sensitivity of the developed method for protein detection was scrutinised using purified protein standards, including Bovine Serum Albumin (BSA) and Fetuin (**Figure 2.11**). These are frequently employed as protein standards across diverse biochemical assays (**Figure 2.11A**). These standards were utilised to construct a calibration curve and ascertain the limit of detection (LOD) inherent to our method. Complementing the use of purified protein standards, the engagement of bacterial protein in the sensitivity evaluation presented a more realistic scenario for tracing microbial protein (**Figure 2.11B**). Bacterial protein for this study was harvested from an *E.coli* culture of known concentration, offering an opportunity to appraise the sensitivity and specificity of our method concerning microbial protein detection. *E. coli* was cultured and protein extracts were prepared using different homogenisation solvents and treatments (i.e. heating and alkaline and acid). The best approach was in the condition of 100 mM NaOH with heating (**Supplementary Figure 2.1**). Protein extracted from *E. coli* was

also used to spike the soil as a more representative mixture of proteins and extracted spiked soil to do Bradford assay (**Figure 2.11B**). The study's outcomes revealed that our method exhibited substantial linearity, reproducibility, and an impressively low detection threshold (0.2 µg/mg soil spiked). This robust demonstration of sensitivity highlights the method's potential for detecting protein traces even in challenging environments, thus demonstrating its suitability for the envisioned application in planetary exploration missions.

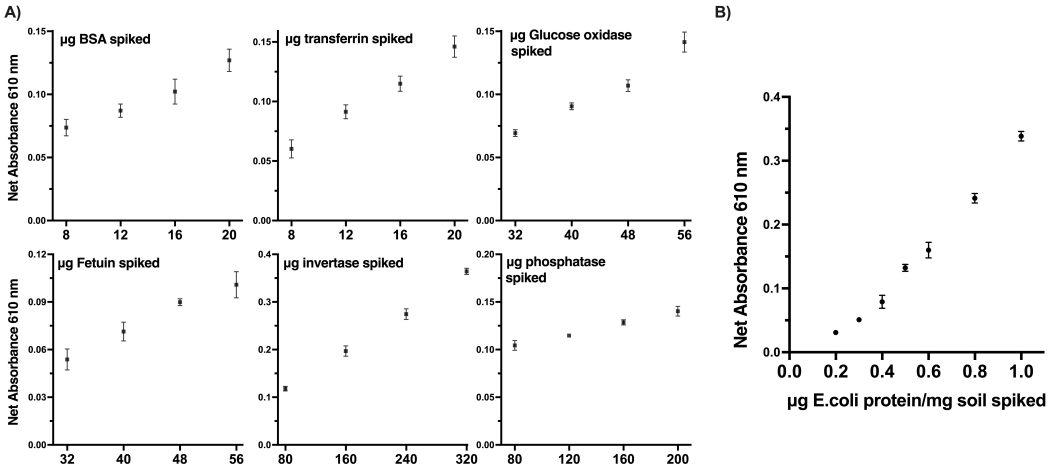


Figure 2.11. Linearity of the protein assay for a series of protein standards and bacterial protein spiked on the soil simulant. Data represent average±SD (N=3).

Finally, to develop this method in platform the option of soil removal, the optimised method was used to spike protein standards of BSA and spiked soils were initially extracted in ddH_2O and soil was removed by filtering or centrifugation (**Figure 2.12**). This illustrated that there was no significant difference between using filtering and centrifugation.

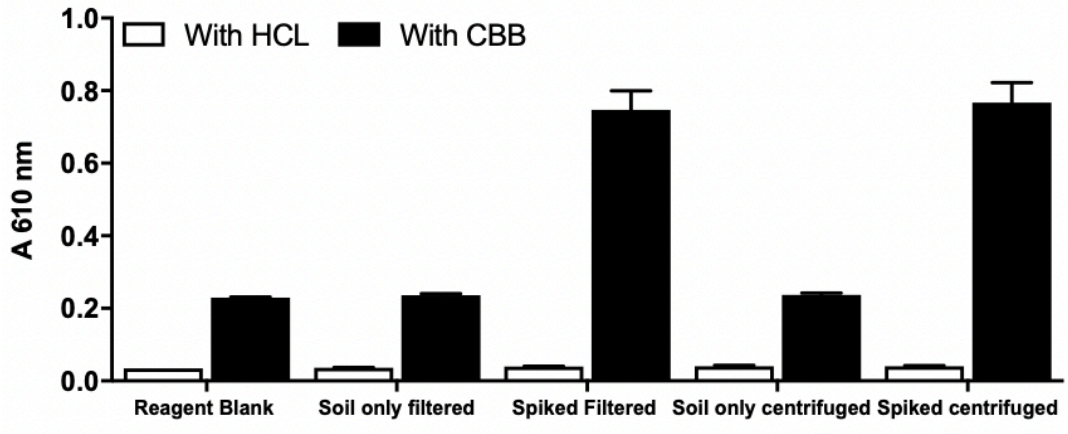


Figure 2.12. Extraction of BSA spiked on soil with ddH_2O and removal of soil debris with filtering or centrifugation. Data represent average±SD (N=3).

2.3.2 The impact of UV radiation and temperature on protein biosignatures

It is expected that ultraviolet (UV) radiation presents formidable challenges to the survival of macromolecules, particularly proteins, within the confines of Martian soil surface. Based on the existing research for the Mars, the data gathered by the Curiosity rover indicated that the total UV radiation dosage at Gale Crater is confined to an upper limit of 20 W/m^2, or 72 kJ/hm^2 (Carrier et al., 2019). In order to simulate the Martian environment and elucidate the influence of UV radiation on proteins, an experimental setup was established where proteins were spiked into soil simulants and subsequently exposed to UV radiation commensurate with the aforementioned dosage of 72 kJ/hm^2 (**Figure 2.13**). The protein BSA was spiked to the soil simulants and then exposed to the UV radiation over a period ranging up to five hours (**Figure 2.13A**). Following they were extracted in ddH_2O, and the residual protein content was quantified using the Bradford assay. The experimental findings indicated a significant reduction in signal following a five-hour UV exposure period, especially after five hours exposure, the signal decreased for 55.02% (**Figure 2.13B**). This diminution in signal detection underscores the deleterious effects of UV radiation on proteins embedded within the Martian soil simulant. This can most likely be ascribed to the UV-induced degradation and fragmentation of these proteins, thereby reinforcing the challenges posed by UV radiation to the maintenance and detection of biological macromolecules on Mars.

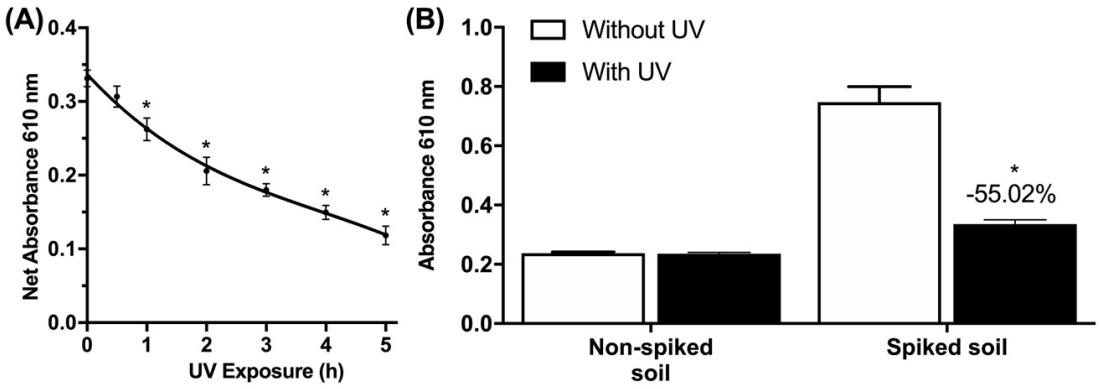


Figure 2.13. The impact of UV radiation to protein spiked on soil. (A) Time-dependent UV exposure effect on BSA-spiked soil samples. The figure depicts the gradual decline in detectable protein concentration, as measured by the Bradford assay, with increasing UV exposure time ranging from 0 to 5 hours. (B) Comparison of protein concentration after 5 hours of UV exposure versus non-exposed control. The figure shows a notable decrease (55.02%) in signal, indicating the degradation of proteins upon UV exposure. Data represent average±SD (N=3) and were considered statistically significant (*) by Student's t-test compared to UV exposure.

In addition to assessing the effects of UV radiation, this study also probed the thermal stability of proteins. Due to the very thin atmosphere of Mars, it almost no capacity to store heat during the day which can cause the great difference in day versus night temperature (Fairén, 2010). The temperature on Mars is relatively low, averaging about minus 60 degrees Celsius. A summer day on Mars may get up to 20 degrees C near the equator, but at night the temperature can plummet to about minus 73°C. It can drop to an average of -80°C at the poles during winter and can even reach 125°C (Leovy, 2001). In this case, BSA alone and BSA-spiked soil samples were exposed to temperatures of -18°C and -80°C, simulating the wide range of cold temperatures on Mars from equatorial regions to the poles (**Figure 2.14**).

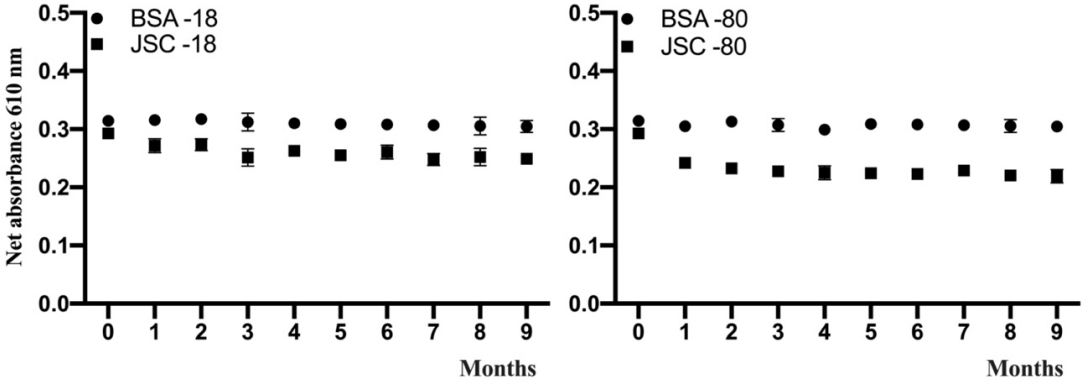


Figure 2.14. Thermal stability of BSA in extreme cold conditions. The figure presents the resilience of both isolated BSA and BSA spiked-soil samples under temperatures of -18°C and -80°C, representative of Mars' harsh cold climate. Data represent average±SD (N=3).

2.3.3. Optimizations for consideration for a platform development

Our data revealed a resilience of BSA to these extreme temperature conditions. Both in its isolated form and when spiked into the soil, BSA demonstrated a high degree of stability and robustness. Irrespective of the temperature, whether it be -18°C or an even more severe -80°C, the protein remained stable, and its structural integrity was largely preserved. These findings provide encouraging implications for the potential persistence and detectability of proteins within the harsh and extreme thermal environment of Mars. It underscores the fact that, while UV radiation poses substantial challenges to protein survival, extreme cold conditions may not necessarily present the same level of threat to protein stability.

A mission to Mars would require 9 months, hence, the CBB reagent was tested for its stability over time stored at -20°C (**Figure 2.15**). Frozen batches of the reagent were compared to freshly prepared samples, both in pre-plated microwell plates and in bulk aliquots. Samples of 25 µl of CBB reagent, 50 µl of CBB:2M HCl reagent, or aliquots of either of these solutions were periodically thawed and assessed for their sensitivity compared to the freshly prepared CBB reagent. Results revealed a time-dependent decrease in the protein detection signal in the microplate stored dye, with the CBB

reagent showing a gradual decrease of 25% after 12 months, while the CBB:2M HCl reagent showed a more significant decrease of 65% after the same storage period. However, the aliquoted reagents remained stable over the 12-month period. These findings suggest that the CBB reagent can be stored in preloaded frozen compartments for future planetary missions, indicating its potential as a reliable method for the detection of proteins as potential biomarkers of life in extraterrestrial environments.

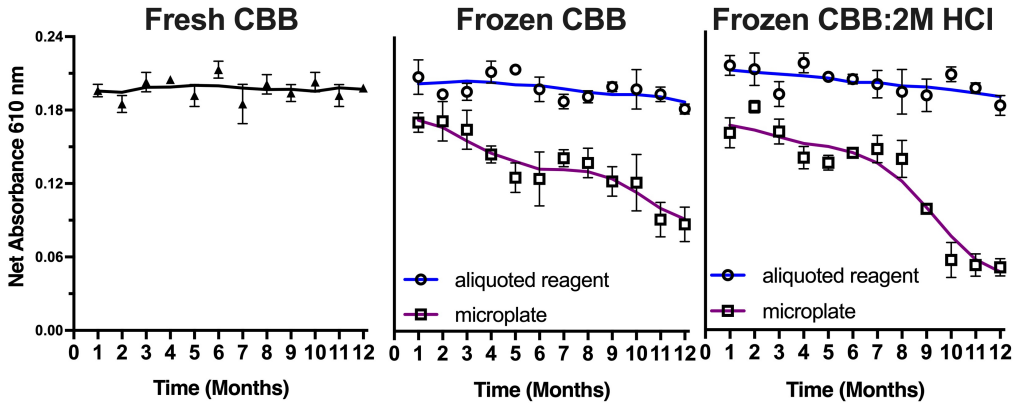


Figure 2.15. Stability of the CBB reagent over storage time. CBB was stored as aliquots of the CBB reagent frozen mixed with equal volume of 2M HCl frozen, in aliquots (open circles) or frozen on well plates (closed squares). Data represent average±SD (N=3).

2.4 Discussion

To identify proteins in Martian soil, the preferred method should possess excellent sensitivity and reproducibility, minimal susceptibility to contamination and interferences, and the ability to withstand harsh conditions and environments until a sample can be tested (de Vera, 2020). Till now, there has not been any instrument for the detection of life directly on any of the Mars exploration missions since the Viking in the 1970s, which hinders any discovery for the existence of life on Mars whenever it may have happened in the past or present (Maggiori et al., 2020). As one of the biomolecules related to the origin of life, proteins are a valuable target biosignature for life detection. In the context of exploration of life existence on Mars, the development of a sensitive and reliable method to identify these life-related proteins accurately could be a promising technique of election for Mars exploration.

The optimised Bradford assay was based on the research of Grintzalis et al. (2015), which provided a simple and quick approach to detect proteins. The traditional Bradford assay needed not only Coomassie Brilliant Blue G-250 but also the kosmotropic and protein precipitating reagents (Georgiou et al., 2008). However, the complexities associated with these reagents pose significant challenges when attempting to detect proteins in space during exoplanet missions, requiring a multitude of reagents for successful implementation. Consequently, our optimized and simplified version of the assay integrates only two essential reagents: CBB and HCl. This simplified approach offers several advantages, such as reduced costs, improved preparation time, and enhanced sensitivity in protein detection. By eliminating the need for superfluous reagents, our optimized assay fulfils the aforementioned criteria and demonstrates promise as a straightforward quantitative method for protein detection in soil samples. Furthermore, in contrast to the traditional Bradford assay, which relies on Coomassie Brilliant Blue G-250, kosmotropic agents, and protein precipitating reagents (Georgiou et al., 2008), our finalized protocol solely necessitates a filtered CBB reagent diluted with 2M HCl, ensuring stability over an extended period.

One of the main problems to detect proteins in soil was that there could be many

interfering substances pre-existing in soil. Generally, there has been a series of approaches to extract proteins from soils and several caveats have been highlighted. In the study of Chen et al. (2009), a sequential extraction method was designed to remove the main interfering substances. The target soil was extracted in citrate and SDS buffers sequentially and finally extracted by phenol to recover the protein (Chen et al., 2009). This method made it possible to get the applicable 1-D and 2-D protein profiles as well as found the glomalin-related soil protein. Although, this was an efficient protocol for soil proteomics, however, it may not be suitable to be operated in space, or in the other words, it will require many efforts and face many challenges to achieve the goal as the crucial environment in space. Our optimised Bradford assay can avoid many interfering substances from soil. By using centrifugation to extract the protein-spiked soil in ddH_2O, it will remove most debris and obtain clear supernatant. Following, the assay was applied to compare the non-spiked soil, and the result showed the linearity for the detection of proteins without any interferences as the relevant control group (using non-spiked soil) was clear and without interfering compounds. This proved that the soil itself will not affect the accuracy of the protein-detection.

The isotope method is another potent strategy proposed for protein detection in extra-terrestrial environments. This technique exploits the distinct isotopic composition of reactants, which influences chemical and biochemical reaction dynamics. Recent research has highlighted the concept of isotopic resonance as a factor that can catalyse reaction kinetics (Xie and Zubarev, 2015). Given that isotopic composition of elements like carbon, hydrogen, nitrogen, and oxygen can display variance between biotic and abiotic systems, a comprehensive isotopic analysis of a sample might enable the isotope method to discern life-related signatures. However, this approach carries its own set of limitations. For instance, it necessitates stringent control over the isotopic composition of reactants, which may prove challenging, if not unfeasible, under the volatile and austere conditions synonymous with extra-terrestrial environments (Ader et al., 2016). Moreover, its effectiveness in detecting proteins per se is doubtful, given that its primary focus lies in assessing the influence of isotopic composition on reaction

kinetics. Therefore, within the context of protein detection in alien environments like Mars, the Bradford assay emerges as a more viable option. This stems from its simplicity, extensive dynamic range, and compatibility with a wide array of sample types, rendering it a practical and flexible tool for potential astrobiological applications.

Furthermore, the Signs of Life Detector (SOLID) is also an advanced tool, conceived for the exploration and characterization of organic carbon on Mars. This instrument leverages the technologies of liquid extraction and lab-on-a-chip immunoassay techniques (Parro et al., 2011). Since its inception in 2000, SOLID has seen substantial technological advancements. At its core is the LDChip biosensor, armed with up to 450 antibodies that can interact with biological materials including proteins, sugars, and DNA. This allows it to detect signatures of life, whether contemporary or ancient (Parro et al., 2018). Notably, there are parallels between the LDChip biosensor within SOLID and the Bradford assay used for protein detection in this study. Both techniques are grounded in the principle of molecular binding to discern the presence of target entities, a finding that can serve as a beacon of potential life. Despite these similarities, however, the Bradford assay maintains an edge due to its simplicity, user-friendliness, and wide-ranging applicability to diverse samples. It also offers a broad dynamic range, contributing to its practicality. On the other hand, the LDChip biosensor demands complex handling and processing steps, encompassing the addition of specific reagents and antibodies. This complexity escalates the chances of errors and contamination (Garcia-Descalzo et al., 2019). Furthermore, the LDChip analysis requires specialized laboratory facilities and trained personnel for proper operation and interpretation of results. In contrast, the Bradford assay is less resource-intensive, enabling execution in a standard laboratory setting, making it a more accessible choice for a wide spectrum of research environments.

Another advantage of our Bradford assay was that it could also detect the organic proteins. Due to the complex and extreme environment on Mars, there were many hypotheses about what life look likes on Mars. One of them was iron-oxidizing bacteria (Floyd et al., 2019). These bacteria belong to chemolithoautotrophic organisms which

can survival without oxygen and use Fe(II) as an electron donor (Floyd et al., 2019). Another organism displaying resilience in extreme Martian-like conditions is the halophilic archaeon Hvr (Feshangsaz et al., 2020). Studies reveal that Hvr exhibits formidable resistance to radiation and desiccation under conditions simulating outer space. In our adapted Bradford assay, proteins extracted from E. coli were detected with a high sensitivity of 0.2 μg protein/mg soil spiked, bolstering the argument that the method could potentially detect proteins from a wide variety of microbial sources. It is essential to clarify that, while E. coli is not identical to the bacteria and archaea hypothesized to exist under Mars-like conditions, it serves as a well-characterized and widely accepted model organism, given its extensive study (Pontrelli et al., 2018). Using E. coli protein as a positive control is commonplace in microbiological research due to its potential for providing a standardized, reproducible benchmark in assessing methodological performance. Consequently, our study lays a robust groundwork for the potential application of this protein extraction method in detecting microbial proteins within extra-terrestrial environments.

UV radiation is a major component when describing extreme conditions on Mars and it has the capacity to inflict irreversible cellular damage, resulting in cell death (Horneck, 1999). Besides UV radiation, gamma-radiation is also used as representative of cosmic radiation in testing its destructive effect on biosignature analogues. In our research, we considered the effects of UV radiation on Martian proteins, as the generation of potent oxidants via UV radiation is identified as a primary catalyst for protein degradation (Fornaro et al., 2020). The decision to focus on UV radiation was also informed by the existing body of knowledge. There is a wealth of data on the effects of gamma radiation on biological molecules, but comparatively less comprehensive research on UV radiation. Thus, this study aimed to contribute to bridging this gap, offering insights into the specific impacts of UV radiation on biosignature analogues. The magnitude of UV radiation on Mars is subject to considerable fluctuation, contingent upon aspects such as geographical location, time of day, and seasonal variations. Consequently, we utilized a UV dosage approximating the total UV radiation dosage at Gale Crater,

measured at 72 kJ/hm^2 (Carrier et al., 2019). The UV radiation employed spanned the spectrum from 280 to 400 nm, encompassing UVA and UVB radiation, known for their capacity to damage proteins, DNA, and other biological molecules. Within our Bradford assay, soil spiked with proteins was subjected to direct UV radiation over various durations, and the resulting absorbance was subsequently gauged. The discerned decline in absorbance with increased UV radiation exposure duration suggests the degradation of the spiked proteins due to UV radiation. This finding aligns with the outcomes of the BIOMEX (BIOlogy and Mars EXperiment) study. The BIOMEX experiment aimed to investigate the effects of space and Mars-analogous conditions on a diverse array of organisms, including methanogenic archaea, fungi, mosses, cyanobacteria, and lichens. The results revealed that all organisms displayed some measure of survival, physiological activity, and growth capability; nevertheless, their vital functions exhibited varying levels of decline under simulated space or Martian conditions. Of note, methanogenic archaea demonstrated a higher resistance compared to multicellular organisms like fungi and lichens (de Vera et al., 2019).

Despite the encouraging outcomes of our research, we must recognize several constraints intrinsic to our assays. Initially, our study did not explore the impacts of perchlorates, cosmic rays, and other potential protein degradation catalysts on our protein detection methods. Particularly, perchlorates, discovered in Martian soil, have proven to adversely affect microbial viability. Further research is necessitated to ascertain the potential influence of these factors on our protein detection assays, particularly in the ambit of planetary exploration. Secondly, our assays were designed with Earth-native proteins in mind. It remains plausible that Martian proteins, should they exist, might comprise a distinct amino acid repertoire, thereby influencing their detectability using our methods. While the fundamental chemistry of protein detection remains consistent across varying amino acid compositions, we cannot disregard the potential inapplicability of our assays to Martian proteins. This underscores the need for in-depth exploration into the biochemistry of Martian lifeforms and the formulation of assays capable of discerning a wider spectrum of protein structures.

In light of potential variances in the chemical composition of planetary soils, the acidic or alkaline nature of these substrates must be acknowledged as a significant variable in the context of biosignature detection. The acidic pH of Bradford assay could be altered when exposed to alkaline soils, a factor that warrants comprehensive examination. The interactive effect between the soil's pH and the assay's effectiveness is a nuanced aspect that could potentially influence the sensitivity and specificity of protein-like biosignature identification. For instance, an alkaline soil environment could potentially neutralize the acidic pH of the Bradford assay, thereby impacting the colorimetric response and, by extension, the accuracy of protein quantification. To address this, future iterations of this research could involve a systematic evaluation of the Bradford assay's performance across a spectrum of soil pH levels. Implementing a pH adjustment step or exploring alternative assays resilient to pH fluctuations might be essential to ensure the reliability of biosignature detection in diverse extraterrestrial environments. This adaptation could bolster the assay's applicability and accuracy, providing a more holistic and adaptable approach to detecting protein-like biosignatures in a variety of planetary soils.

The aforementioned processes can be seamlessly integrated within a miniaturized, autonomous platform, such as the MICRO-life detection platform, capable of conducting real-time analysis of DNA, RNA, proteins, and other smaller molecules (Fernández-Martínez et al.), or incorporated into larger missions, especially if pre-aliquoted reagents are stored in vessels or well plates ready for utilization. The domain of protein chemistry within soil matrices is undergoing comprehensive scientific scrutiny, with an increasing number of research articles pivoting towards proteomic approaches for protein identification in soil.

The quest for life on Mars has received renewed impetus following the discovery of a potentially habitable environment in Gale Crater's sedimentary record. An array of techniques, including Elemental Analysis-Isotope Ratio Mass Spectrometry, Laser-ablation Ionisation Mass Spectrometry, Raman spectroscopy, and Fourier Transform InfraRed spectroscopy, was employed to analyze a simulated Martian mudstone

material inoculated and cultured in an attempt to detect biosignatures. Though high-sensitivity techniques managed to retrieve presumptive biosignatures, the sedimentary matrix presented obstacles for all techniques, suggesting that definitive evidence for life might be challenging to obtain (Stevens et al., 2019). While our approach does not provide identification outcomes (as it wasn't the intent), this method could hold substantial relevance not only in astrobiology but also in broader disciplines such as geosciences and agriculture. Offering a cost-effective, portable method with a wide dynamic range, compatibility with a variety of sample types, and ease of execution, it could be utilized directly in the field to evaluate soil quality in terms of its protein content.

2.5 Conclusions

The focal point of this chapter was the meticulous refinement of an economical and sensitive biochemical procedure for protein quantification, using the Bradford-based assay as a base and tailored for application to Martian soil simulants. The optimisation of this method necessitated a comprehensive exploration of various parameters of the assay, including spiking volume, extraction time, and the concentration of spiking protein.

Notably, the strategic use of methanol as a spiking agent, combined with the evaporation of the spike solution through a speed vacuum concentrator or oven, emerged as an optimised technique. Further investigations revealed negligible differences among varying spiking volumes, concentrations of spike, and extraction times. These collective findings paved the way for the development of an efficient, optimised method tailored to the research objectives.

An additional layer of testing involved bacterial protein, specifically from *E.coli*, serving as a representative of potential microbial life. The method demonstrated effectiveness irrespective of whether filtering or centrifugation was employed to separate the soil from its extract. Thus, the final protocol provides an economical, streamlined, and efficient approach to detect proteins in simulated Martian soil, showing versatility and adaptability.

Furthermore, consideration was given to time constraints and the stability of reagents, with steps taken to freeze the working dye and explore alternatives for its dissolution. These additional measures improve the method's suitability for future planetary missions by ensuring the stability and longevity of critical reagents.

In conclusion, this chapter has outlined the optimisation of a cost-effective and sensitive protein quantification method. The developed technique holds promising implications for astrobiology, especially in enhancing our capability to detect potential biosignatures, thereby propelling the understanding of the universe and life's place within it.

2.6 References

ADER, M., THOMAZO, C., SANSJOFRE, P., BUSIGNY, V., PAPINEAU, D., LAFFONT, R., CARTIGNY, P. & HALVERSON, G. P. 2016. Interpretation of the nitrogen isotopic composition of Precambrian sedimentary rocks: Assumptions and perspectives. *Chemical Geology,* 429**,** 93-110.

AGUIRRE, J. 2023. *The Kjeldahl Method: 140 Years*, Springer Nature Switzerland.

BADA, J. L., GLAVIN, D. P., MCDONALD, G. D. & BECKER, L. 1998. A search for endogenous amino acids in martian meteorite ALH84001. *Science,* 279**,** 362-5.

BAINS, W. 2004. Many chemistries could be used to build living systems. *Astrobiology,* 4**,** 137-67.

CARRIER, B. L., ABBEY, W. J., BEEGLE, L. W., BHARTIA, R. & LIU, Y. 2019. Attenuation of Ultraviolet Radiation in Rocks and Minerals: Implications for Mars Science. *Journal of Geophysical Research: Planets,* 124**,** 2599-2612.

CHEN, S., RILLIG, M. C. & WANG, W. 2009. Improving soil protein extraction for metaproteome analysis and glomalin-related soil protein detection. *Proteomics,* 9**,** 4970-3.

CHEVALLET, M., LUCHE, S. & RABILLOUD, T. 2006. Silver staining of proteins in polyacrylamide gels. *Nat Protoc,* 1**,** 1852-8.

COMPTON, S. J. & JONES, C. G. 1985. Mechanism of dye response and interference in the Bradford protein assay. *Anal Biochem,* 151**,** 369-74.

DE VERA, J.-P. 2020. Biomolecules in Space: The Way to Search for Life on Mars. *Astrobiology on the International Space Station.*

DE VERA, J. P., ALAWI, M., BACKHAUS, T., BAQUE, M., BILLI, D., BOTTGER, U., BERGER, T., BOHMEIER, M., COCKELL, C., DEMETS, R., DE LA TORRE NOETZEL, R., EDWARDS, H., ELSAESSER, A., FAGLIARONE, C., FIEDLER, A., FOING, B., FOUCHER, F., FRITZ, J., HANKE, F., HERZOG, T., HORNECK, G., HUBERS, H. W., HUWE, B., JOSHI, J., KOZYROVSKA, N., KRUCHTEN, M., LASCH, P., LEE, N., LEUKO, S., LEYA, T., LOREK, A., MARTINEZ-FRIAS, J., MEESSEN, J., MORITZ, S., MOELLER, R.,

OLSSON-FRANCIS, K., ONOFRI, S., OTT, S., PACELLI, C., PODOLICH, O., RABBOW, E., REITZ, G., RETTBERG, P., REVA, O., ROTHSCHILD, L., SANCHO, L. G., SCHULZE-MAKUCH, D., SELBMANN, L., SERRANO, P., SZEWZYK, U., VERSEUX, C., WADSWORTH, J., WAGNER, D., WESTALL, F., WOLTER, D. & ZUCCONI, L. 2019. Limits of Life and the Habitability of Mars: The ESA Space Experiment BIOMEX on the ISS. *Astrobiology,* 19**,** 145-157.

DOMON, B. & AEBERSOLD, R. 2006. Mass spectrometry and protein analysis. *Science,* 312**,** 212-7.

FAIRÉN, A. G. 2010. A cold and wet Mars. *Icarus,* 208**,** 165-175.

FERNÁNDEZ-MARTÍNEZ, M. Á., O'CONNOR, B., BOURDAGES, L.-J., MAGGIORI, C. & WHYTE, L.

FESHANGSAZ, N., SEMSARHA, F., TACKALLOU, S. H., NAZMI, K., MONAGHAN, E. P., RIEDO, A. & VAN LOON, J. 2020. Survival of the Halophilic Archaeon Halovarius luteus after Desiccation, Simulated Martian UV Radiation and Vacuum in Comparison to Bacillus atrophaeus. *Orig Life Evol Biosph,* 50**,** 157-173.

FLOYD, M. A. M., WILLIAMS, A. J., GRUBISIC, A. & EMERSON, D. 2019. Metabolic Processes Preserved as Biosignatures in Iron-Oxidizing Microorganisms: Implications for Biosignature Detection on Mars. *Astrobiology,* 19**,** 40-52.

FORNARO, T., BRUCATO, J. R., POGGIALI, G., CORAZZI, M. A., BICZYSKO, M., JABER, M., FOUSTOUKOS, D. I., HAZEN, R. M. & STEELE, A. 2020. UV Irradiation and Near Infrared Characterization of Laboratory Mars Soil Analog Samples. *Frontiers in Astronomy and Space Sciences,* 7.

GARCIA-DESCALZO, L., PARRO, V., GARCIA-VILLADANGOS, M., COCKELL, C. S., MOISSL-EICHINGER, C., PERRAS, A., RETTBERG, P., BEBLO-VRANESEVIC, K., BOHMEIER, M., RABBOW, E., WESTALL, F., GABOYER, F., AMILS, R., MALKI, M., MARTEINSSON, V., VANNIER, P., EHRENFREUND, P., MONAGHAN, E., RIEDO, A., CABEZAS, P., WALTER,

N. & GOMEZ, F. G. 2019. Microbial Markers Profile in Anaerobic Mars Analogue Environments Using the LDChip (Life Detector Chip) Antibody Microarray Core of the SOLID (Signs of Life Detector) Platform. *Microorganisms,* 7.

GEORGIOU, C. D., GRINTZALIS, K., ZERVOUDAKIS, G. & PAPAPOSTOLOU, I. 2008. Mechanism of Coomassie brilliant blue G-250 binding to proteins: a hydrophobic assay for nanogram quantities of proteins. *Anal Bioanal Chem,* 391**,** 391-403.

GILLESPIE, A. W., FARRELL, R. E., WALLEY, F. L., ROSS, A. R. S., LEINWEBER, P., ECKHARDT, K.-U., REGIER, T. Z. & BLYTH, R. I. R. 2011. Glomalin-related soil protein contains non-mycorrhizal-related heat-stable proteins, lipids and humic materials. *Soil Biology and Biochemistry,* 43**,** 766-777.

GRINTZALIS, K., GEORGIOU, C. D. & SCHNEIDER, Y. J. 2015. An accurate and sensitive Coomassie Brilliant Blue G-250-based assay for protein determination. *Anal Biochem,* 480**,** 28-30.

HAWE, A., SUTTER, M. & JISKOOT, W. 2008. Extrinsic fluorescent dyes as tools for protein characterization. *Pharm Res,* 25**,** 1487-99.

HORNECK, G. 1999. European activities in exobiology in Earth orbit: Results and perspectives. *Advances in Space Research,* 23**,** 381-386.

HURISSO, T. T., MOEBIUS‐CLUNE, D. J., CULMAN, S. W., MOEBIUS‐CLUNE, B. N., THIES, J. E. & ES, H. M. 2018. Soil Protein as a Rapid Soil Health Indicator of Potentially Available Organic Nitrogen. *Agricultural & Environmental Letters,* 3.

JOHN SAVORY, PIN H. PU & SUNDERMAN, F. W. 1968. A Biuret Method for Determination of Protein in Normal Urine. *Clinical Chemistry,* 14**,** 1160-1171.

JULL, A. J., COURTNEY, C., JEFFREY, D. A. & BECK, J. W. 1998. Isotopic evidence for a terrestrial source of organic compounds found in martian meteorites Allan Hills 84001 and Elephant Moraine 79001. *Science,* 279**,** 366-9.

KIMBERLY, A. E. & ROBERTS, M. G. 1905. A Method for the Direct Determination

of Organic Nitrogen by the Kjeldahl Process. *Public Health Pap Rep,* 31**,** 109-22.

KUMAR, A., KUJUR, A., SINGH, P. P. & PRAKASH, B. 2019. Nanoencapsulated plant-based bioactive formulation against food-borne molds and aflatoxin B(1) contamination: Preparation, characterization and stability evaluation in the food system. *Food Chem,* 287**,** 139-150.

LADNER, C. L., YANG, J., TURNER, R. J. & EDWARDS, R. A. 2004. Visible fluorescent detection of proteins in polyacrylamide gels without staining. *Anal Biochem,* 326**,** 13-20.

LEOVY, C. 2001. Weather and climate on Mars. *Nature,* 412**,** 245-9.

LOWRY, O. H., ROSEBROUGH, N. J., FARR, A. L. & RANDALL, R. J. 1951. Protein measurement with the Folin phenol reagent. *J Biol Chem,* 193**,** 265-75.

MAGGIORI, C., STROMBERG, J., BLANCO, Y., GOORDIAL, J., CLOUTIS, E., GARCIA-VILLADANGOS, M., PARRO, V. & WHYTE, L. 2020. The Limits, Capabilities, and Potential for Life Detection with MinION Sequencing in a Paleochannel Mars Analog. *Astrobiology,* 20**,** 375-393.

MCKAY, D. S., GIBSON, E. K., JR., THOMAS-KEPRTA, K. L., VALI, H., ROMANEK, C. S., CLEMETT, S. J., CHILLIER, X. D., MAECHLING, C. R. & ZARE, R. N. 1996. Search for past life on Mars: possible relic biogenic activity in martian meteorite ALH84001. *Science,* 273**,** 924-30.

MILO, R. & PHILLIPS, R. 2015. *Cell Biology by the Numbers*.

NASA. 2005. *Partial Ingredients for DNA and Protein Found Around Star* [Online]. Available: https://www.nasa.gov/vision/universe/starsgalaxies/spitzer-20051220.html [Accessed February 12 2020].

NIELSEN, S. S. 2021. Correction to: Food Analysis Fifth Edition. *Food Analysis.*

O'FARRELL, P. H. 1975. High resolution two-dimensional electrophoresis of proteins. *The Journal of biological chemistry,* 250**,** 4007-4021.

OGUNSEITAN, O. A. 2006. Soil Proteomics: Extraction and Analysis of Proteins from Soils. *In:* NANNIPIERI, P. & SMALLA, K. (eds.) *Nucleic Acids and Proteins in Soil.* Berlin, Heidelberg: Springer Berlin Heidelberg.

PARRO, V., BLANCO, Y., PUENTE-SANCHEZ, F., RIVAS, L. A., MORENO-PAZ, M., ECHEVERRIA, A., CHONG-DIAZ, G., DEMERGASSO, C. & CABROL, N. A. 2018. Biomarkers and Metabolic Patterns in the Sediments of Evolving Glacial Lakes as a Proxy for Planetary Lake Exploration. *Astrobiology,* 18**,** 586-606.

PARRO, V., DE DIEGO-CASTILLA, G., RODRIGUEZ-MANFREDI, J. A., RIVAS, L. A., BLANCO-LOPEZ, Y., SEBASTIAN, E., ROMERAL, J., COMPOSTIZO, C., HERRERO, P. L., GARCIA-MARIN, A., MORENO-PAZ, M., GARCIA-VILLADANGOS, M., CRUZ-GIL, P., PEINADO, V., MARTIN-SOLER, J., PEREZ-MERCADER, J. & GOMEZ-ELVIRA, J. 2011. SOLID3: a multiplex antibody microarray-based optical sensor instrument for in situ life detection in planetary exploration. *Astrobiology,* 11**,** 15-28.

PATTON, W. F. 2000. A thousand points of light: The application of fluorescence detection technologies to two-dimensional gel electrophoresis and proteomics. *Electrophoresis,* 21**,** 1123-1144.

PONTRELLI, S., CHIU, T. Y., LAN, E. I., CHEN, F. Y., CHANG, P. & LIAO, J. C. 2018. Escherichia coli as a host for metabolic engineering. *Metab Eng,* 50**,** 16-46.

REDMILE-GORDON, M. A., ARMENISE, E., WHITE, R. P., HIRSCH, P. R. & GOULDING, K. W. 2013. A comparison of two colorimetric assays, based upon Lowry and Bradford techniques, to estimate total protein in soil extracts. *Soil Biol Biochem,* 67**,** 166-173.

SCHWIETERMAN, E. W., KIANG, N. Y., PARENTEAU, M. N., HARMAN, C. E., DASSARMA, S., FISHER, T. M., ARNEY, G. N., HARTNETT, H. E., REINHARD, C. T., OLSON, S. L., MEADOWS, V. S., COCKELL, C. S., WALKER, S. I., GRENFELL, J. L., HEGDE, S., RUGHEIMER, S., HU, R. & LYONS, T. W. 2018. Exoplanet Biosignatures: A Review of Remotely Detectable Signs of Life. *Astrobiology,* 18**,** 663-708.

SEAGER, S. 2014. The future of spectroscopic life detection on exoplanets. *Proc Natl Acad Sci U S A,* 111**,** 12634-40.

STEVENS, A. H., MCDONALD, A., DE KONING, C., RIEDO, A., PRESTON, L. J., EHRENFREUND, P., WURZ, P. & COCKELL, C. S. 2019. Detectability of biosignatures in a low-biomass simulation of martian sediments. *Sci Rep,* 9**,** 9706.

TOMANEK, L. 2014. Proteomics to study adaptations in marine organisms to environmental stress. *J Proteomics,* 105**,** 92-106.

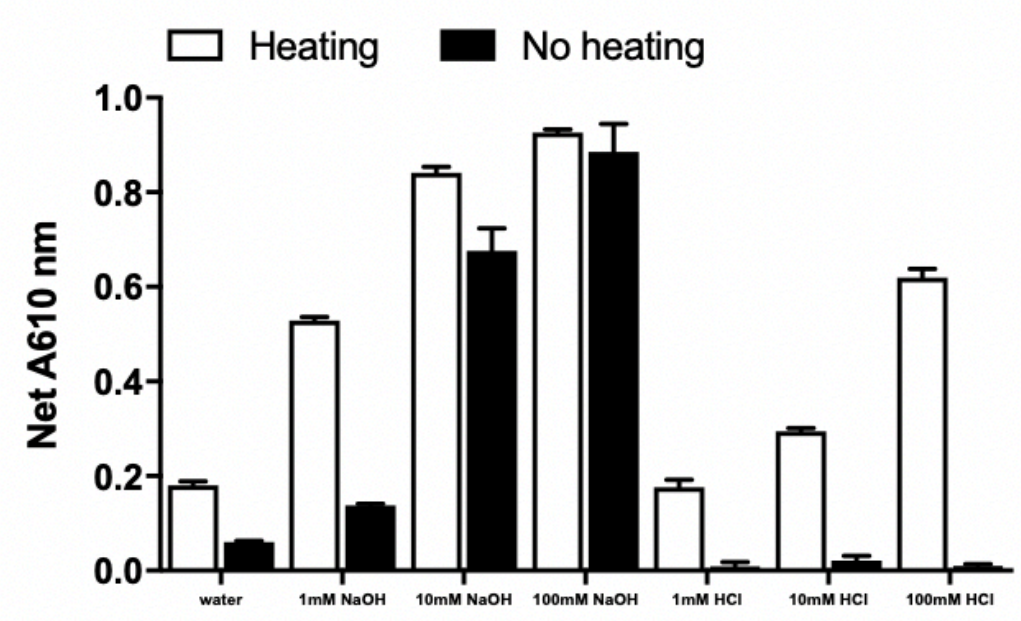
WASHBURN, M. P., WOLTERS, D. & YATES, J. R., 3RD 2001. Large-scale analysis of the yeast proteome by multidimensional protein identification technology. *Nat Biotechnol,* 19**,** 242-7.

WRIGHT, S. F. & UPADHYAYA, A. 1996. Extraction of an Abundant and Unusual Protein from Soil and Comparison with Hyphal Protein of Arbuscular Mycorrhizal Fungi. *Soil Science,* 161**,** 575-586.

WU, Q., YUAN, H., ZHANG, L. & ZHANG, Y. 2012. Recent advances on multidimensional liquid chromatography-mass spectrometry for proteomics: from qualitative to quantitative analysis--a review. *Anal Chim Acta,* 731**,** 1-10.

XIE, X. & ZUBAREV, R. A. 2015. Isotopic resonance hypothesis: experimental verification by Escherichia coli growth measurements. *Sci Rep,* 5**,** 9215.

Supplementary Figures



Supplementary Figure 2.1. Different homogenisation solvents and treatments (i.e. heating and alkaline and acid). Data represent average ± SD (N = 3).

Chapter 3: Development of methods to detect nucleic acids as biosignatures of extra-terrestrial life

Abstract

Nucleic acids are the biomolecules that are the carriers of genetic information integral to life, and as such they could serve as potential biosignatures in the quest for extra-terrestrial existence. Research into nucleic acids as target molecules in the exploration of life's origins is a vibrant and evolving field. For instance, a NASA-funded project recently synthesized DNA comprising all eight nucleotides as a novel approach to life detection. Concurrently, the Search for Extra-Terrestrial Genomes (SETG) instrument was developed to extract and sequence nucleic acids on Mars using nanopore technology. Despite the promise, these high-cost missions are not immune to failure. Hence, developing simpler, highly specific, conclusive assays with a minimal step procedure could mitigate potential risks and bolster the effectiveness of these exploratory tests. This chapter delves into the development and application of a straightforward method for quantifying DNA on Martian soil simulants using fluorescent dyes. Further, the potential impact of UV radiation, a likely stressor on Martian surface, on the stability and detectability of DNA was assessed. This research underscores the importance of thorough pre-mission testing and paves the way for more reliable life-detection strategies in our quest to uncover life beyond Earth.

3.1 Introduction

3.1.1 The Evolution of nucleic acids

Nucleic acids, as one of the essential biomolecules, carry the genetic information of organisms. Nucleic acids refer to deoxyribonucleic acid (DNA) and ribonucleic acid (RNA), which consist of nucleotides (Lundblad and Macdonald, 2018)with DNA composed of deoxynucleotides and RNA of ribonucleotides. Deoxynucleotides consist of the sugar deoxyribose, phosphate groups and the option between four nitrogenous bases: adenine (A), guanine (G), thymine (T) and cytosine (C). The structure of DNA molecular consists of two polydeoxynucleotide antiparallel chains which are coiled around a common central axis to form a double helix structure. The deoxyribose-phosphate chain is outside the helix, with the bases facing inside. The two polydeoxynucleotide chains are reverse complementary, connected by hydrogen bonds between the nitrogenous bases, forming a fairly stable combination. In the genomes of organisms, genetic information is stored in DNA sequences, and the transmission of this genetic information is ensured by the presence of complementary nitrogen-containing base sequences. In fact, during transcription, genetic information can be easily transcribed into complementary RNA molecules with one category known as messenger RNA (mRNA) which translates information to proteins (Lundblad and Macdonald, 2018).

RNA molecules exist in cells and some viruses and viroid as genetic material. Similar to DNA, a ribonucleotide molecule is composed of phosphoric acid, ribose and nitrogenous bases. There are four main bases of RNA, namely adenine (A), guanine (G), cytosine (C), and uracil (U) which replaces thymine (T) in DNA (Lundblad and Macdonald, 2018). There are three general types of RNA: transfer RNA (tRNA), messenger RNA (mRNA), and ribosomal RNA (rRNA). All three of the aforementioned participate in the process of translation; with the mRNA acting as a “messenger” molecule transferring the genetic information the tRNA molecules bringing the amino acids for protein synthesis and the rRNAs as components of the ribosomes (Glick et al., 2010). In addition, many other kinds of functional RNA are now known, such as microRNAs, small nuclear RNAs etc. (Gebert and MacRae, 2019).

The emergence of life on Earth is believed to be rooted in the molecular pathways of RNA, a theory supported by evidence that traces the constituents of early RNA polymers to meteoritic origins (Pearce et al., 2017). The hypothesis is that there was a hypothetical prebiotic species on the early Earth which had a different base sequence from any in known genomes (Neveu et al., 2013). The existence of the ribosome, which can be considered as a ribozyme, could support

this hypothesis. Although the ribosome is an assemblage of RNA and protein components, the RNA moieties are primarily responsible for its critical function of translation. This suggests that in Earth's early biosphere, RNA's catalytic capabilities likely held precedence over protein-based mechanisms (ExploringOrigins, 2020). In terms of molecular evolution, DNA is posited to have emerged from its RNA predecessor, a transformation thought to have initially transpired within viral entities before propagating to cellular life (Forterre, 2001). The advent of DNA was not an isolated event but was underpinned by a constellation of enzymatic activities, which facilitated critical biochemical processes, including synthesis of DNA precursors, retro-transcription of RNA templates, and replication of both single and double-stranded DNA molecules (Forterre et al., 2004). With its inception, DNA has had a profound influence on the trajectory of life on Earth. The universal presence of double-stranded DNA genomes across all cellular organisms today underscores its paramount importance in shaping the biosphere as we perceive it.

3.1.2 Nucleic acids as biosignatures

Although it is unknown whether any extra-terrestrial life form will be similar to the one on Earth, it is valuable to start to research on biosignatures which exist on Earth as a reference for extra-terrestrial life. Biomolecules, such as nucleic acids, proteins and carbohydrates, are essential components of life, which can be considered as the biosignature for searching life beyond Earth (Summons et al., 2007).

DNA serves as the blueprint of life on Earth, providing the instructions that guide the development, function, growth, and reproduction of all known living organisms. At the molecular level, DNA is a complex, double-stranded structure composed of sequences of four nucleotide bases: adenine, guanine, cytosine, and thymine (Minchin and Lodge, 2019). The specific arrangement of these bases constitutes the genetic code, encoding the information necessary for the synthesis of proteins and the regulation of their activity.

Within the cell, DNA undergoes transcription to produce messenger RNA (mRNA), which in turn undergoes translation, guiding the assembly of amino acids into proteins within the ribosomes (Crick, 1970). This central dogma of molecular biology – DNA to RNA to protein – underlies the processes of life as we understand them, from the simplest of bacteria to the most complex of eukaryotes.

Beyond its role in protein synthesis, DNA is critical for the inheritance of traits (Martin and Zhang, 2007). Through the process of replication, the information encoded in a parent cell's

DNA is passed onto its progeny, thereby preserving the genetic continuity across generations. DNA's stability, capacity for storing complex information, and self-replication potential are key factors that have allowed life to thrive and diversify on Earth.

With DNA's profound influence on terrestrial life, one could reasonably speculate that DNA, or a similar molecule, might form the basis of life in extra-terrestrial settings (Devine and Jheeta, 2020). Firstly, the fundamental chemistry of DNA is rooted in elements that are common throughout the universe. Carbon, hydrogen, oxygen, nitrogen, and phosphorus – the elemental constituents of nucleic acids – are not exclusive to Earth but are found abundantly in cosmic environments (Irwin and Schulze-Makuch, 2011). This raises the possibility that similar molecular structures may have arisen elsewhere under suitable conditions. Secondly, the structure and function of DNA exhibit a robustness and versatility that could be advantageous in a variety of environments (Travers and Muskhelishvili, 2015). The double-helix provides a level of stability, while the ability of nucleotide sequences to encode information is highly flexible. In principle, any environment that allows for the existence of stable, information-encoding polymers could potentially support life as we know it, or even life as we don't yet know it (Lutz, 2020). Finally, the phenomenon of convergence, where disparate evolutionary lineages arrive at similar solutions to common problems, suggests that life – if it exists elsewhere – may have converged on a DNA-like solution for the storage and transfer of genetic information (Powell, 2012). While these considerations are certainly speculative, they provide a basis for the search for DNA as a biosignature of extraterrestrial life, a search that will not only test these hypotheses but also expand our understanding of life's possibilities.

Researching for meteorites could be used to trace the origin of life on the early Earth. Among all kinds of meteorites, carbonaceous chondrite meteorites are considered as the important research object as they contain carbonaceous materials which are essential for terrestrial life (Pizzarello, 2007). These materials can be divided into soluble compounds and insoluble macromolecules. For instance, the Murchison meteorite, one of the carbonaceous chondrite meteorites fell in Australia in 1969, have been carried out extensive research by scientists, and showed that in soluble compounds, nucleic acid bases, purines and pyrimidines were detected but no ribose (Callahan et al., 2011, Cavalazzi and Westall, 2019). Several lines of evidence indicate that the interior portions of well-preserved fragments from Murchison are pristine, ruling out the possibility of terrestrial contamination (Joseph, 2023). According to the Studies focusing on life on Mars and the meteorite transfer between Mars and Earth, it showed that nucleic acids, as the biological informational polymers, could be a worthwhile research subject

to prove the life existence on Mars (Mojarro et al., 2019). The Search for Extra-Terrestrial Genomes (SETG) life-detection instrument was designed recently by Mojarro et al. (2019) to extract nucleic acids on Mars and then perform nanopore sequencing. This assay can determine the genetic sequence from samples on Mars to compare with the life on Earth to see if there is a shared ancestry between Mars and Earth.

3.1.3 Methods for the detection and quantification of nucleic acids

The common method to detect and quantify nucleic acids in laboratory rely on optical technology. Based on their intrinsic absorption properties, nucleic acids can be detected by absorbance at 260 nm using spectrophotometers. Another approach is using fluorogenic dyes to combine with DNA or RNA to generate a fluorescence signal which can be measured by fluorometers (Pflugradt et al., 2012, Gallagher and Desjardins, 2008).

There are several advantages using fluorescence. First of all, according to the specificity of different fluorogenic dyes, different dyes can be used to detect DNA, ds DNA, ssDNA and RNA explicating selectivity (Holden et al., 2009). Fluorescence in general as an approach has higher sensitivity to detect nucleic acids, and the result can be more accurate and stable with less risk for contamination of samples (Gallagher and Desjardins, 2008, Holden et al., 2009). In this study, the PicoGreen dye is used to quantify DNA extracted from soil samples. PicoGreen is a fluorescent dye which can combine with double stranded DNA (dsDNA) and forms a highly luminescent complex (Dragan et al., 2010). This dye is more specific to detect dsDNA with no interfering from proteins. Additional controls and optimisation were performed to establish a robust and conclusive approach.

According to the RNA world hypothesis, searching the trace of the evolution of RNA is also important. To prove this hypothesis, it is important to develop a technology to verify the biological characteristics of the hypothetical sequence. In the research of Wasik et al. (2019), decision algorithms were proposed which based on the normalized compression distance (NCD) and Levenshtein distance (LD), to detect signatures of life in short RNA samples as the life probe. Although both NCD and LD have the similar capability to distinguish life signatures, LD works well when using the reference set with the same distribution of nucleotides as in biological sequences whereas NCD cannot distinguish them (Wasik et al., 2019).

Another method proposed to detect life signals was creating informatics fingerprints which based on DNA sequencing (Johnson et al., 2018). This approach relies on the detection of sequences of nucleic acid from a wide variety of life and combine them as a fingerprint. The

fingerprint can be used to detect life signals by analysing the molecular patterns and complexity. The advantage of the fingerprint was that it can be used to detect the life even it is not nucleic acid-based, which means the life vastly different from Earth can be also detected (Johnson et al., 2018).

3.1.4 Challenges and limitations in detecting nucleic acids for planetary missions

Executing advanced methodologies in the field of astrobiology, specifically in planetary missions, entails addressing an array of complex challenges. One such concern is the potential for contamination, a risk that persists despite sterilization procedures (Green et al., 2023). Terrestrial nucleic acids could accidentally contaminate the spacecraft, its instruments, or the collected samples, thus raising the spectre of false-positive detections of extra-terrestrial life. This concern spans multiple stages of the mission, from the initial assembly of the spacecraft to the collection, preservation, and subsequent analysis of samples (Raymond-Bouchard et al., 2022). To mitigate these risks, stringent protocols would need to be devised, including the establishment of cleanroom facilities for assembly, comprehensive pre-launch sterilization of all equipment, and the implementation of contamination control measures during the various stages of sample collection and analysis (Beauchamp and Belz, 2013).

Additionally, the extreme conditions likely to be encountered in extra-terrestrial environments can pose considerable obstacles to the detection of nucleic acids. Factors such as high levels of ionizing radiation, drastic temperature variations, and the presence of chemical compounds potentially harmful to nucleic acids could impede detection efforts (Le Vay et al., 2020). These harsh conditions may also impact the operational capabilities and lifespan of the instruments deployed for detection and analysis. Thus, a significant engineering challenge lies in the design and fabrication of resilient systems capable of withstanding such formidable conditions.

Another intellectual challenge arises in the interpretation of the data, should nucleic acids be successfully detected. If detected sequences bear similarity to those on Earth, it may suggest a shared origin, hinting at panspermia, or alternatively, indicate a convergent evolutionary process where separate origins of life have adopted analogous molecular mechanisms (Demets, 2012). Distinguishing between these possibilities will necessitate a diverse body of corroborative evidence. Conversely, the detection of novel nucleic acid structures or sequences could imply the existence of an entirely distinct form of life, thereby challenging our existing biological paradigms and necessitating the development of interpretive frameworks (Bowater and Brazda, 2022).

Furthermore, the advanced techniques utilized for nucleic acid detection and analysis, such as Next-Generation Sequencing, often rely on the controlled conditions of a laboratory environment, replete with extensive infrastructure and resources (Slatko et al., 2018). Such conditions are presently unattainable within a spacecraft setting due to the complex nature of sample preparation, the heavy computational demands, and the sheer volume of data generated. Overcoming these logistical hurdles will undoubtedly require substantial technological innovation and the successful miniaturization of existing equipment.

3.1.5 Structure of the Chapter

This chapter presents a focused discussion on nucleic acids, with particular emphasis on the detection of DNA as biosignatures for extra-terrestrial life by using fluorescent dyes. The primary objective of this research is to develop and optimize a novel, sensitive, and cost-effective method for detecting and quantifying DNA in simulated Martian soil. The method is centred around the use of fluorescent dyes which can provide a robust and practical solution for DNA detection in extreme conditions. This chapter details the optimization process of this method, its practical implementation in Martian soil simulants, and the results obtained. The chapter then investigates how Martian environmental factors like UV radiation affect DNA stability. Through simulated Martian soil spiking and UV radiation exposure, the research evaluates the viability of DNA detection under such challenging conditions. The chapter concludes with a summation of the key findings, the novelty and significance of the optimized DNA detection method, and an exploration of potential future research directions. The conclusion serves to tie together the chapter's arguments, contextualizing them within the broader field of astrobiology and the ongoing search for extra-terrestrial life.

3.2 Materials and Methods

3.2.1 Biochemical methods for the quantification of DNA using fluorescent dyes

The main assay used in this chapter for the development of the method was Hoechst, which is a blue fluorescent stain specific for DNA. When bound to DNA, the DNA-dye complex can be excited by ultraviolet light and it emits blue fluorescence at 460 to 490 nm (Garner, 2009). The Hoechst used in this project was the reagent Hoechst 33342 which binds to the region adenine-thymine (A-T) in DNA preferentially. This stain binds into the minor groove of DNA and exhibits distinct fluorescence emission spectra that are dependent on dye-base pair ratios. The original Hoechst 33342 dye is 20 mM aqueous stock solution, on the day of the experiment, it was diluted 4000-fold to 5 μM with PBS in a plastic container to use and a series of DNA standards (0.1-1 μg/ml) were prepared in PBS. A DNA (from salmon testes) was prepared in Tris-EDTA (TE) buffer at 500 μg/ ml (5 mg DNA dissolved in 10 ml TE buffer, kept in the fridge overnight), and on the day of the experiment, it was diluted in PBS to generate a series of DNA standards (0.1-1 μg/ml).

Another fluorescent dye, the Novel Juice, was also considered as an alternative approach. Novel Juice is a non-mutagenic fluorescent reagent which contains three tracking dyes: Bromophenol Blue, Xylene Cyanol FF, and Orange G. It is the most sensitive stain available for detecting double-stranded DNA (dsDNA) (Motohashi, 2019).

The third fluorescent dye which was optimised for the DNA detection was the PicoGreen dye, which is an ultra-sensitive fluorescent nucleic acid stain which binds to double-stranded DNA (dsDNA) to produce detectable fluorescence at 260 nm (the excitation wavelength is 480 nm and the emission wavelength is 520 nm) (ThermoFisherScientific, 2008). The original PicoGreen dye was dissolved in anhydrous dimethyl sulfoxide (DMSO). On the day of the experiment, it was diluted 200-fold with TE prior buffer in a plastic container to use and a series of DNA standards (5-50 ng/ml) were prepared in TE buffer. The samples and standards were assayed for DNA concentration according to **Table 3.1**.

Table 3.1. Hoechst/Novel Juice/PicoGreen assay conditions. The volumes are in µl. RB: Reagent Blank, S: Sample or Standard.		
Reagents	**RB**	**S**
Sample appropriately diluted in PBS or DNA standard in PBS	-	200
PBS	200	-
Hoechst/Novel Juice	50	50

3.2.2 Spiking of soil samples with DNA

Soil simulants (50 mg JSC Mars-1A Martian Regolith Simulant) were incubated with different concentration of DNA solutions in TE buffer and then dried using the incubator at the temperature 30℃, DNA was suspected remain into JSC and then extracted in PBS and quantified by the fluorescent dyes (**Figure 3.1**). To ensure the accuracy, control groups were made without soil as well as without DNA spiked and sonication was also used to the extraction of spiked soil samples. The overall spiking process was optimized and will be described in the Results section.

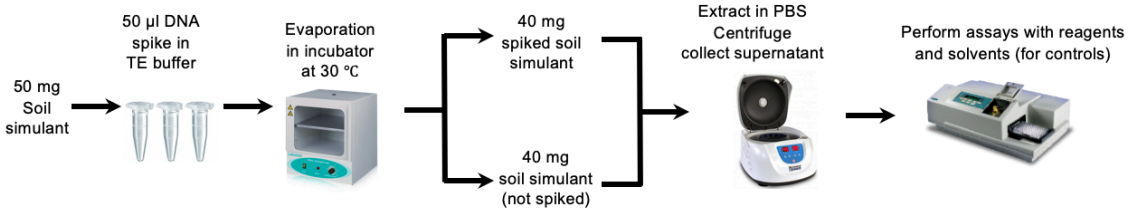


Figure 3.1. Spiking methods of soil samples with DNA.

3.3 Results

A new sensitive approach for the quantification of DNA was established and improved for the spiking and extraction of DNA from a soil simulant matrix. Specific parameters and steps of the novel protocol were optimised as discussed.

3.3.1 Optimisation of the detection and quantification of DNA samples using Hoechst

Initially, focusing on one dye; Hoechst, the impact of the incubation time of the reaction of Hoechst dye and DNA was assessed (**Figure 3.2**). 100 µM of the Hoechst reagent were incubated with DNA for 10 to 60 minutes, and fluorescence was measured (at a gain value of 40). The reaction of Hoechst and DNA was stable up to 60 minutes and the remeasurement will not decrease the sensitivity of fluorescence.

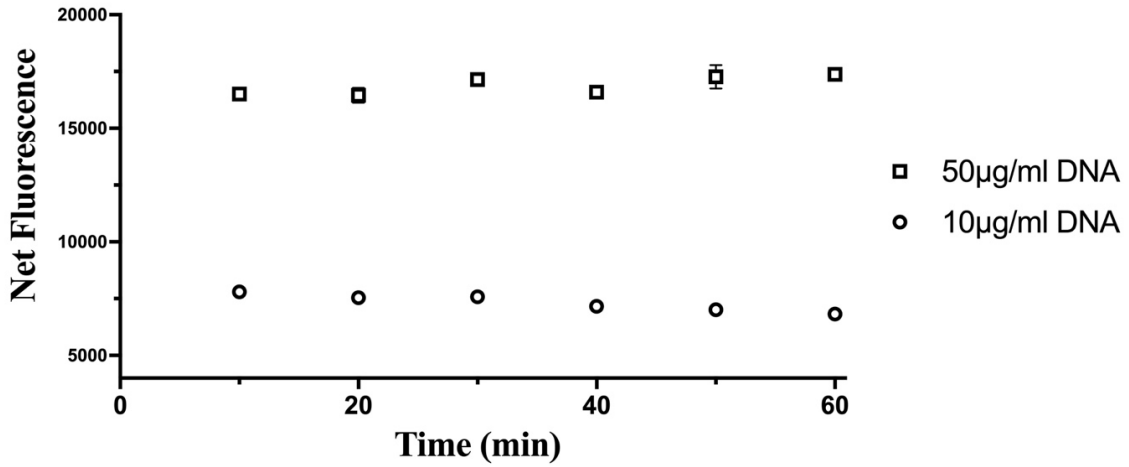


Figure 3.2. The impact of the incubation time by using Hoechst. Data represent average±SD (N=3).

To estimate if remeasuring the same sample would have an impact on its fluorescence, samples were consecutively measured for 20 times (**Figure 3.3**). The results showed that consecutive excitation of the same sample does not impact its fluorescence. This would allow the assay to measure samples (if needed) repeatedly.

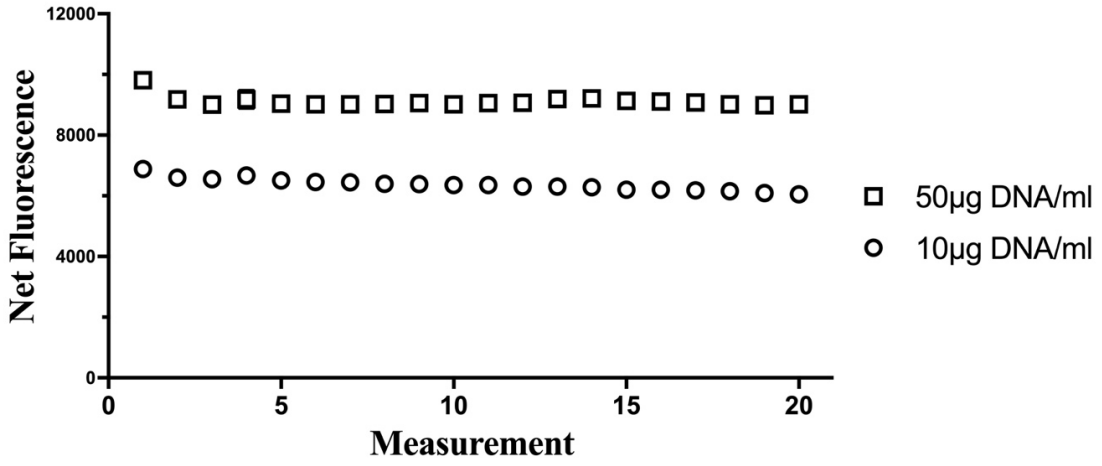


Figure 3.3. The impact of consecutive excitations (remeasuring) of DNA using the Hoechst dye. DNA samples at 10 µg/ml or 50 µg/ml were incubated with 100 µM Hoechst. Data represent average±SD (N=3).

Having established the stability of the reaction, the optimum relationship among DNA concentration, Hoechst concentration and measuring gain (sensitivity of the fluorometer) were explored. In relevance to fluorescence measurements using the Hoechst or any other dye, the plate reader has an option to increase its sensitivity limit via a gain value of the fluorescence. This effectively increases the overall fluorescence since it amplifies the signal measured and, therefore, this would also increase the background fluorescence. A series of concentrations of DNA were incubated with a range of concentrations of Hoechst (in PBS), and fluorescence was measured at different gain values. The slope of the linear DNA standard curve represents the sensitivity of the method (**Figure 3.4A**), while mixtures without DNA (**Figure 3.4B**) represent the background fluorescence of the Hoechst dye (or a reagent blank). An optimal method requires a high sensitivity (high slope) and preferably a low background (reagent blank). These results indicated that with the increase of the DNA concentration, Hoechst concentration and the gain, the fluorescence will also increase, however, the best option is using 5 µM Hoechst and a gain value set at 70, which can measure accurately the DNA standards from 0.1 to 10 µg/ml in PBS.

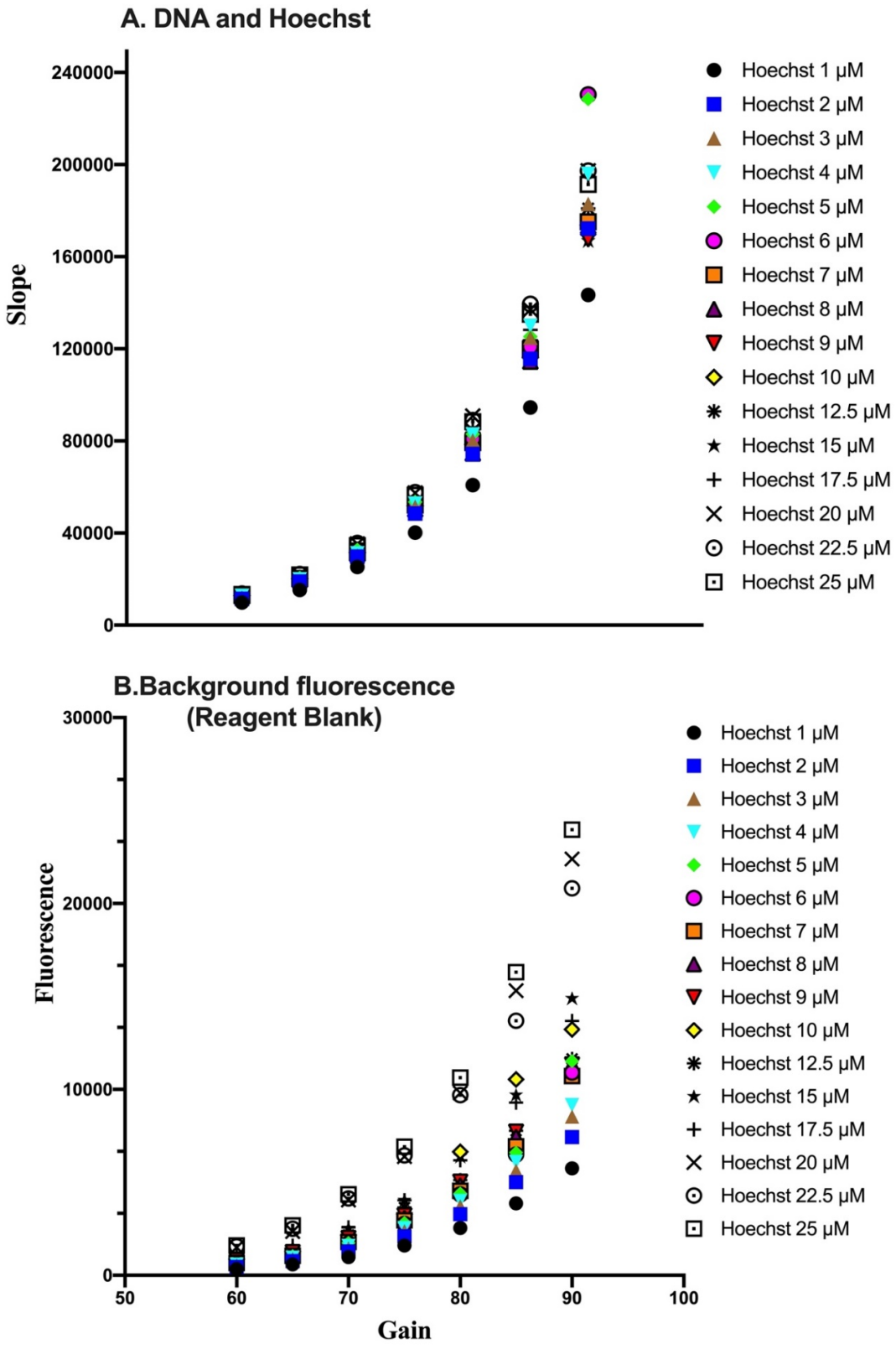


Figure 3.4. The relationship of gain with the sensitivity (DNA standard curve slope) and the bacground (reagent blank) fluorescence for different concentrations of the Hoechst dye. Data represent average±SD (N=3).

Having selected the optimised conditions for the DNA measurements, the linear DNA standard has a limit of detection of minimum 0.1 µg DNA/ml and a very accurate reproducibility (**Figure 3.5**).

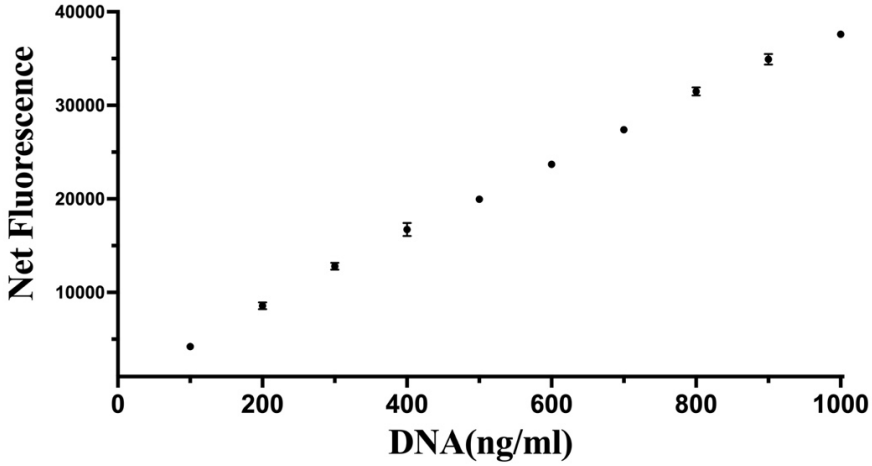


Figure 3.5. Linear standard curve of DNA with 5 µM Hoechst at gain 70. Data represent average±SD (N=3).

3.3.2 Optimisation of the detection and quantification of DNA samples using Novel Juice

The steps for optimizing Novel Juice were similar to the Hoechst. Initially, the fluorescence of different concentration of DNA with Novel Juice was explored for the impact of the incubation time of the reaction (**Figure 3.6**). The Novel Juice dye was diluted 400 times and then mixed with DNA (0.05 or 0.1 µg/ml) and fluorescence was measured up to 60 minutes (at a gain value of 120). The fluorescence signal was stable for 10 mins and then started to decrease, which indicated that the optimum incubation time should not exceed 10 minutes.

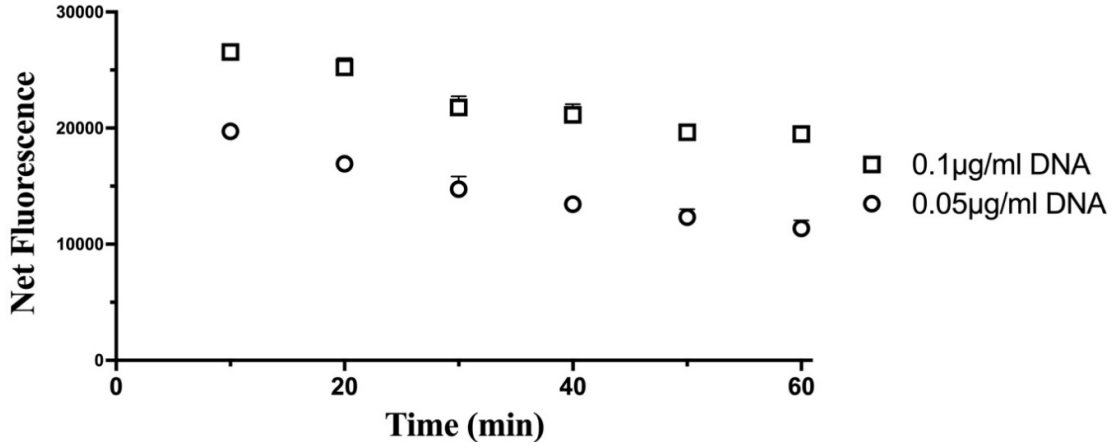


Figure 3.6. The impact of the incubation time by using Novel Juice. Data represent average±SD (N=3).

To estimate the impact of consecutive excitation of the same sample on its fluorescence, samples were measured for 20 times (**Figure 3.7**). The results showed that exciting the same sample several times consequently does not impact its fluorescence and this would allow the assay to measure samples (if needed) repeatedly.

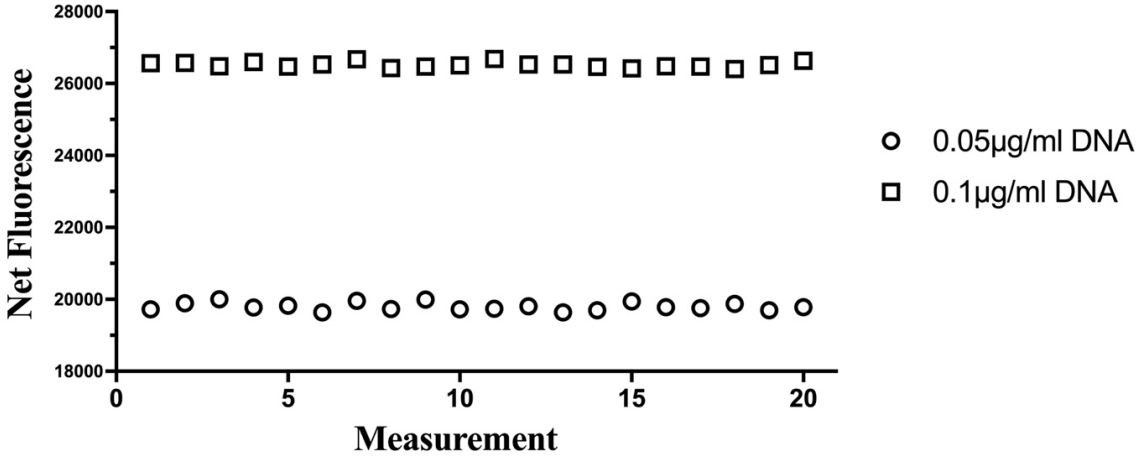


Figure 3.7. The impact of remeasurement of DNA using Novel Juice. DNA at 0.05 µg (circle) or 0.1 µg (square) were incubated with Novel Juice 400 times diluted. Data represent average±SD (N=3).

Following, the concentration of Novel Juice was explored in relation to sensitivity to develop a quantification protocol. The optimization was performed in relevance to the sensitivity of the instrument via different gain values, and similar to previous experiments with Hoechst, the slope of the linear DNA standard curve represents the sensitivity of the method (**Figure 3.8A**), while the absence of DNA (**Figure 3.8B**) represents the background fluorescence of Novel

Juice (reagent blank). As stated before, the optimum region as a compromise between all parameters for a high sensitivity (high slope) and a low background (reagent blank fluorescence was estimated in relation to the concentration of the Novel Juice dye. The optimum gain values would be 90 to 110.

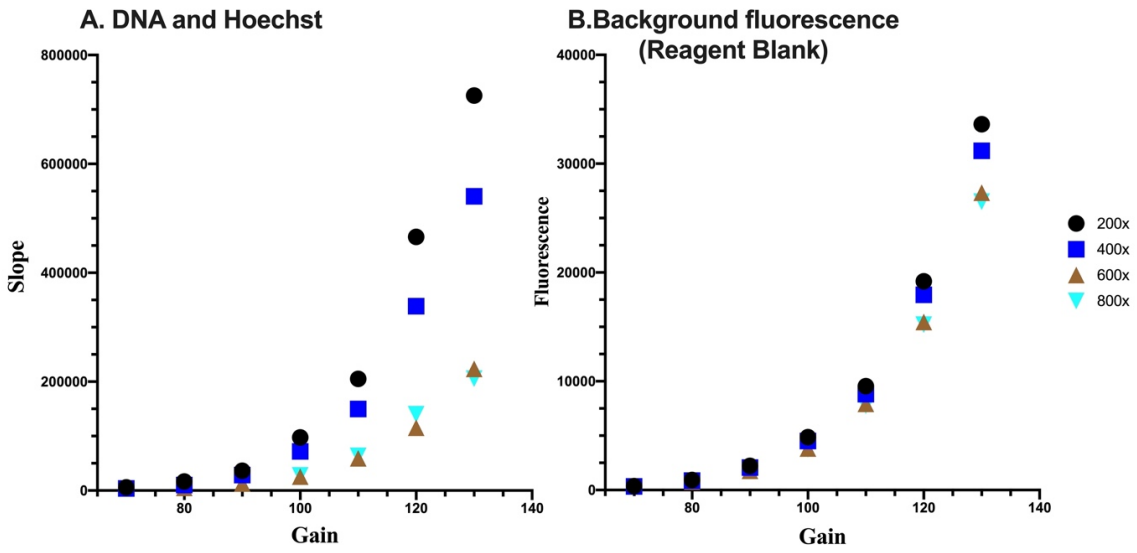


Figure 3.8. The relationship of gain with the sensitivity (DNA standard curve slope) and reagent blank under different concentrations of Novel Juice. Data represent average±SD (N=3).

For the further exploration to determine the best option of the concentration of Novel Juice and the DNA range for the optimum linearity and sensitivity, a range of Novel Juice concentration of reagents was mixed with DNA and the standard curves were plotted to acquire the slopes (**Figure 3.9**). The optimum option is a concentration of 400x dilution of the Novel Juice reagent and a gain value at 100, which could be used measurements of DNA in the range of 0.005 to 0.04 µg/ml in PBS **(Figure 3.10)**.

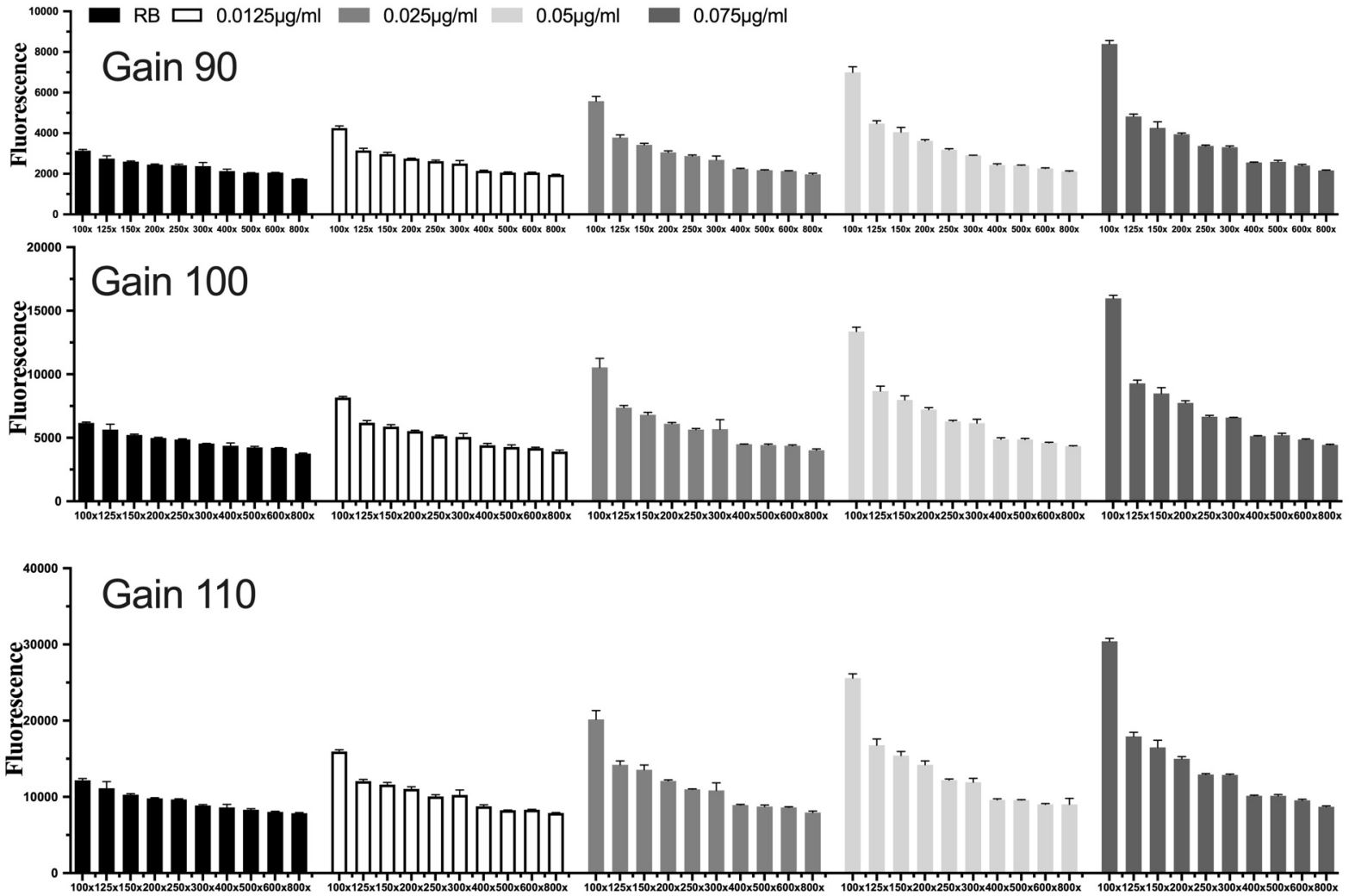


Figure 3.9. the optimisation of the concentration of the Novel Juice dye and gain. Data represent average±SD (N=3).

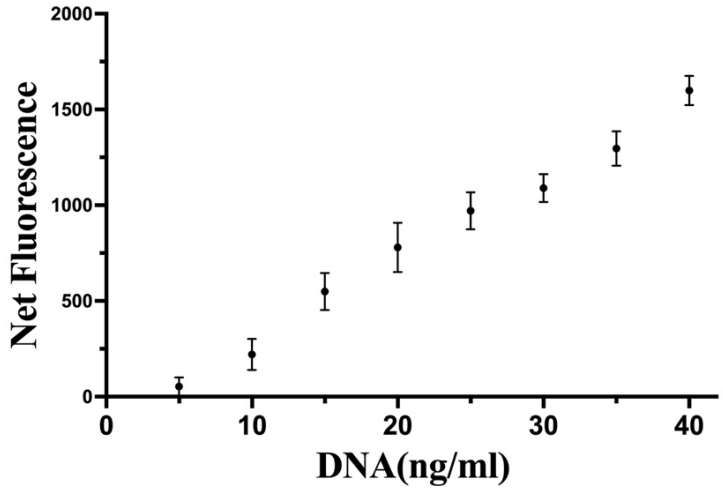


Figure 3.10. Linear standard curve of DNA with 400 times diluted Novel Juice at gain 100. Data represent average±SD (N=3).

3.3.3 Optimisation of the detection and quantification of DNA samples using PicoGreen

In order to explore the optimum reaction conditions of PicoGreen fluorescence dye, the similar steps applied to Hoechst and Novel Juice were also used to PicoGreen. Initially, the fluorescence of different concentration of DNA with PicoGreen was explored for the impact of the incubation time of the reaction (**Figure 3.11**). The PicoGreen dye reacted with DNA (0.05 or 0.1 μg/ml) and fluorescence was measured up to 60 minutes (at a gain value of 54). The fluorescence signal had a slightly decrease at the first 10 minutes and then kept stable. This indicated that the time window for incubating and measuring the fluorescence by using PicoGreen can be up to 60 minutes.

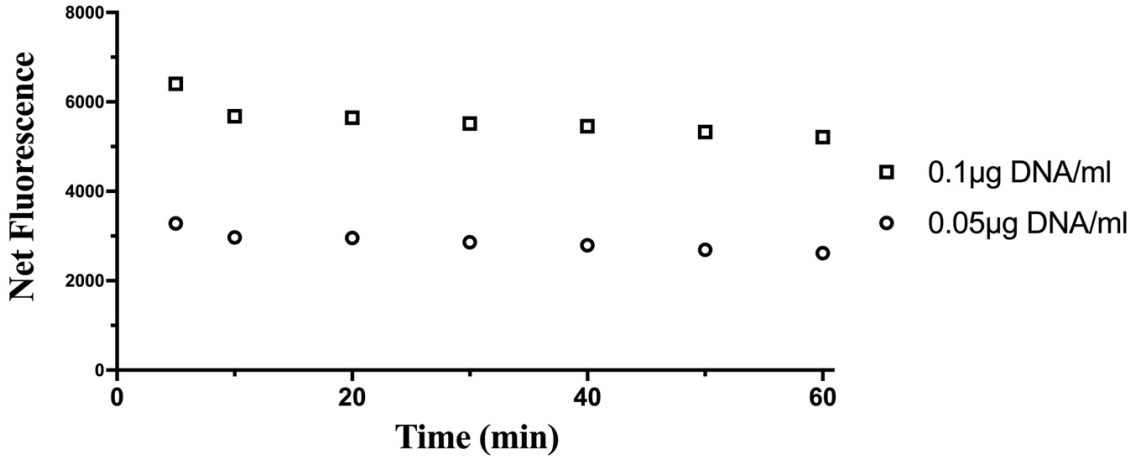


Figure 3.11. The impact of the incubation time by using PicoGreen. Data represent average±SD (N=3).

To estimate the impact of consecutive excitation of the same sample on its fluorescence, samples were measured for 20 times (**Figure 3.12**). The results showed that exciting the same sample does not impact its fluorescence and this would allow the assay to measure samples (if needed) repeatedly.

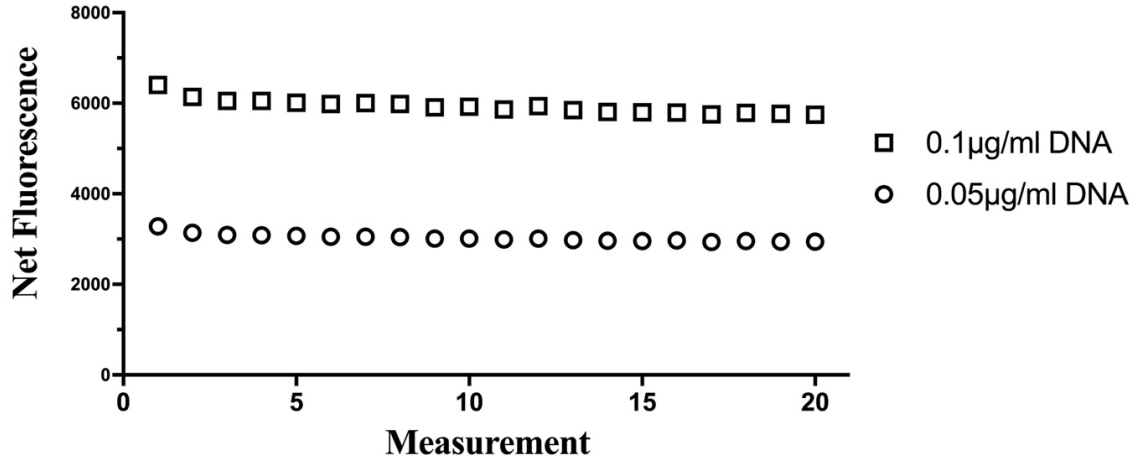


Figure 3.12. The impact of remeasurement of DNA using PicoGreen. DNA at 0.05 µg (square) or 0.1 µg (circle) were incubated with PicoGreen 100 times diluted. Data represent average±SD (N=3).

Following, the concentration of PicoGreen was explored in relation to sensitivity to develop a quantification protocol. The optimization was performed in relevance to the sensitivity of the instrument via different gain values, and similar to previous experiments with Hoechst and Novel Juice, the slope of the linear DNA standard curve represents the sensitivity of the method (**Figure 3.13A**), while the absence of DNA (**Figure 3.13B**) represents the background fluorescence of PicoGreen (reagent blank). As stated before, the optimum region as a compromise between all parameters for a high sensitivity (high slope) and a low background (reagent blank fluorescence was estimated in relation to the concentration of the PicoGreen dye. The optimum gain values would be 70 to 80.

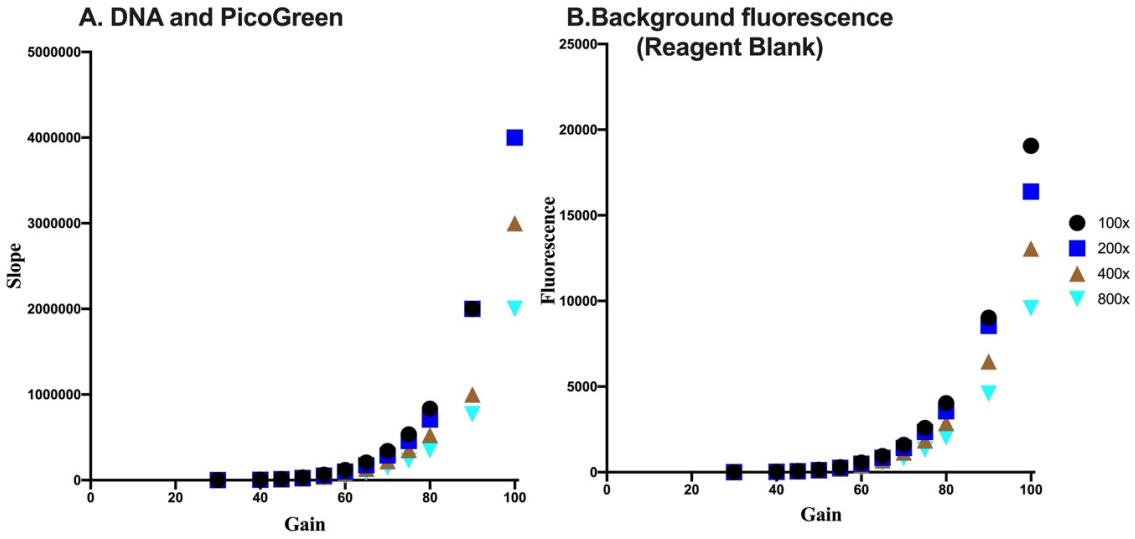


Figure 3.13. The relationship of gain with the sensitivity (DNA standard curve slope) and reagent blank under different concentrations of PicoGreen. Data represent average±SD (N=3).

For the further exploration to determine the best option of the concentration of PicoGreen and the DNA range for the optimum linearity and sensitivity, a range of PicoGreen concentration of reagents reacted with DNA and the standard curves were plotted to acquire the slopes (**Figure 3.14**). The optimum option is a concentration of 400x dilution of the PicoGreen reagent and a gain value at 80, which could be used measurements of DNA in the range of 0.005 to 0.05 µg/ml in PBS **(Figure 3.15)**.

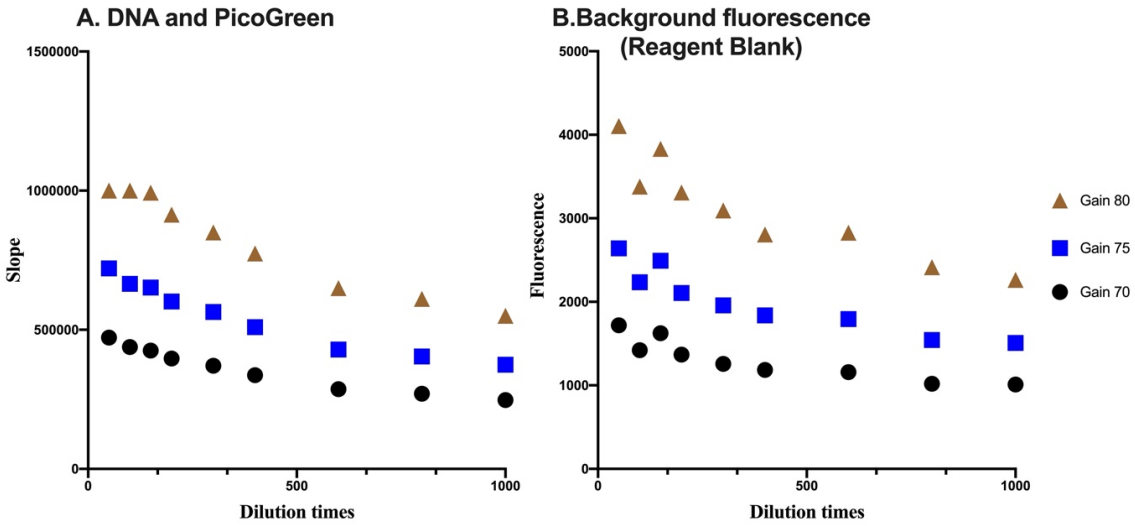


Figure 3.14. the optimisation of the concentration of the PicoGreen dye and gain. Data represent average±SD (N=3).

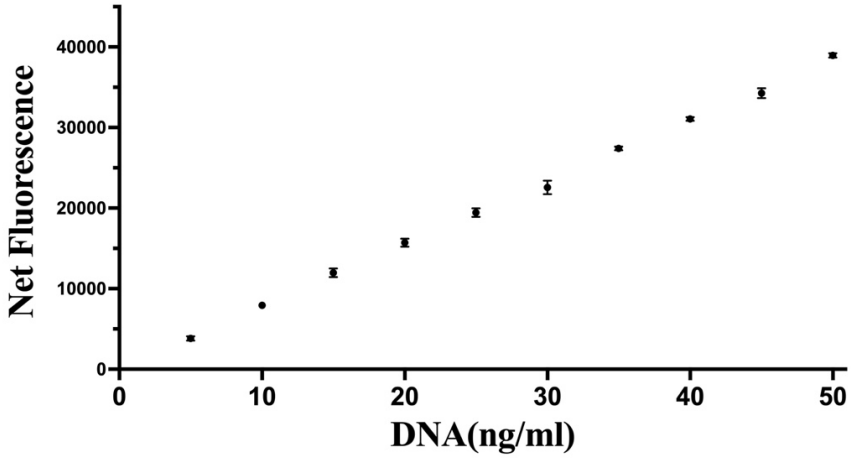


Figure 3.15. Linear standard curve of DNA with 400 times diluted PicoGreen at gain 80. Data represent average±SD (N=3).

3.3.4 Optimisation of the interferences deriving from the soil matrix on DNA measurement

The following steps were optimising using one dye: the Hoechst. Before spiking DNA on the soil, the interference of the soil itself was explored. Soil was used with and without sterilising (by autoclaving) and mixed with DNA. Following, the mixtures were incubated with or without Hoechst (**Figure 3.16**). There was no difference by using sterile soil or non-sterile soil.

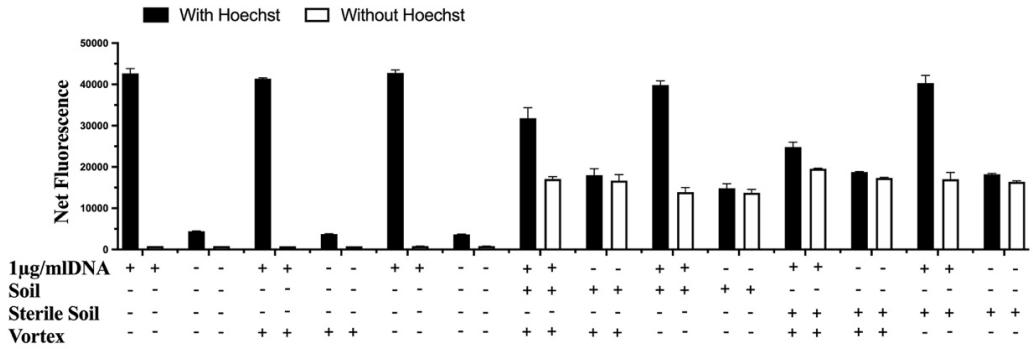


Figure 3.16. The impact of sterile soil on detecting DNA by using Hoechst. Data represent average±SD (N=3).

Following, the extraction volume of PBS on signal interference was assessed using different volume of PBS to extract the soil matrix. Following extraction, the soil extract (extraction supernatant after centrifugation) was collected and incubated with DNA in the presence and absence of Hoechst. This approach would identify any interferences from the soil but without co-existence of DNA and soil, as in this experiment the extract of the soil was obtained in the absence of DNA. The reagent blank comprised of the DNA solution without the presence of soil. The result showed that with the increase of extraction volume, the interference from soil will decrease significantly (**Figure 3.17**).

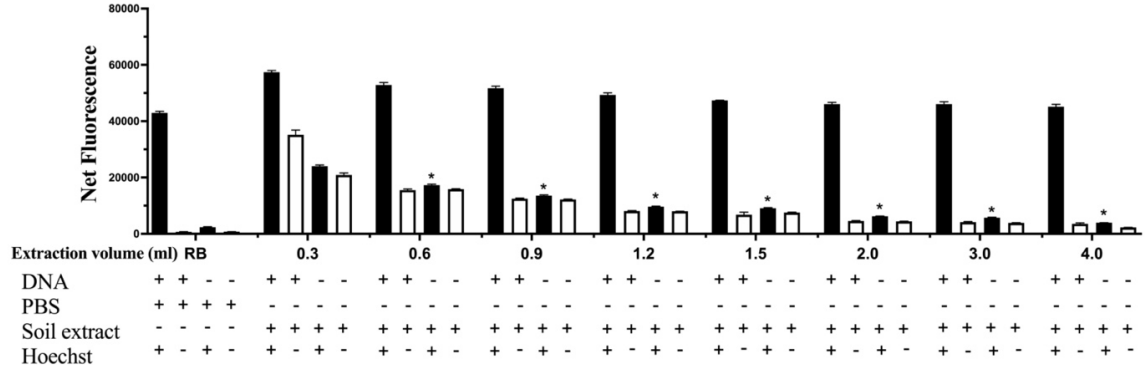


Figure 3.17. The impact of the extraction volume on signal interference due to soil matrix. Data represent average±SD (N=3). *Statistically significant by one-way ANOVA corrected with post-hoc Turkey test.

From the aforementioned, the extraction volume was optimized at 3 ml PBS per 50 mg soil to lower the background of the soil fluorescence. In order to determine if this background affects the DNA directly, 3 ml DNA solution were added directly onto the

soil and extracted (**Figure 3.18**). This approach is effectively simulating the co-incubation of DNA with the soil. The result proved that when the chosen extraction volume (3 ml PBS) decreased successfully the interference from the soil matrix and there is no difference compared with the pure DNA solution in the absence of soil, confirming that this approach does not mask or decrease the fluorescence of DNA. To establish this, the previous experiment (only for 3 ml PBS) was repeated (last four bars).

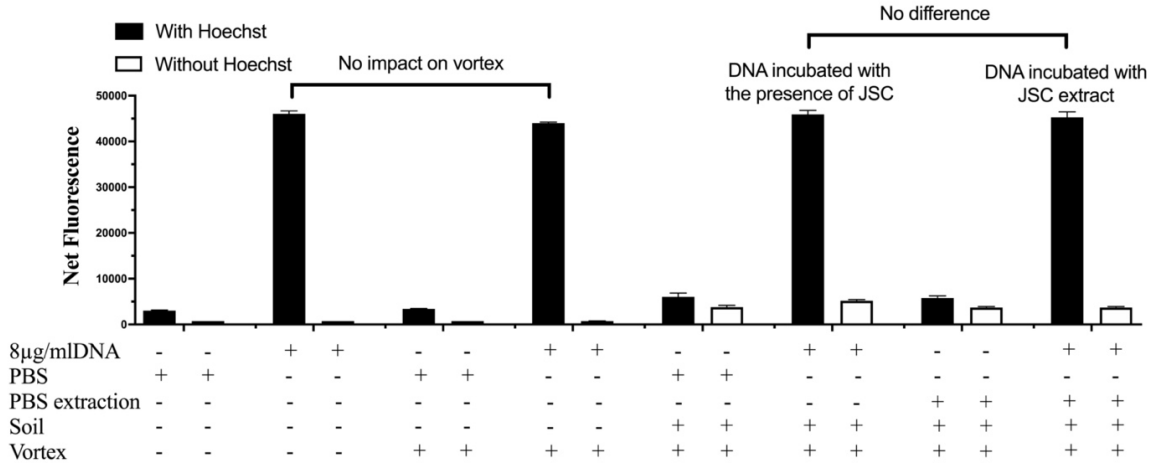


Figure 3.18. The impact of the extraction volume on DNA (1 µg/ml) signal due to interference of the soil matrix. Data represent average±SD (N=3). One-way ANOVA corrected with post-hoc Turkey test was applied and there was no significant difference among each bar.

The alternative approach to remove soil was centrifugation or filtration (**Figure 3.19**). In this case, centrifugation or filtration of pure DNA resulted in no change in the fluorescence signal. However, filtration decreased DNA signal extracted from soil when compared to the blank (without DNA), but this was not the case for centrifugation, therefore, centrifugation would be preferred between the two options. This could be explained because the soil adheres with some DNA and ends up retained by the filter which cannot happen at a low centrifugation speed which would sediment only the soil matrix.

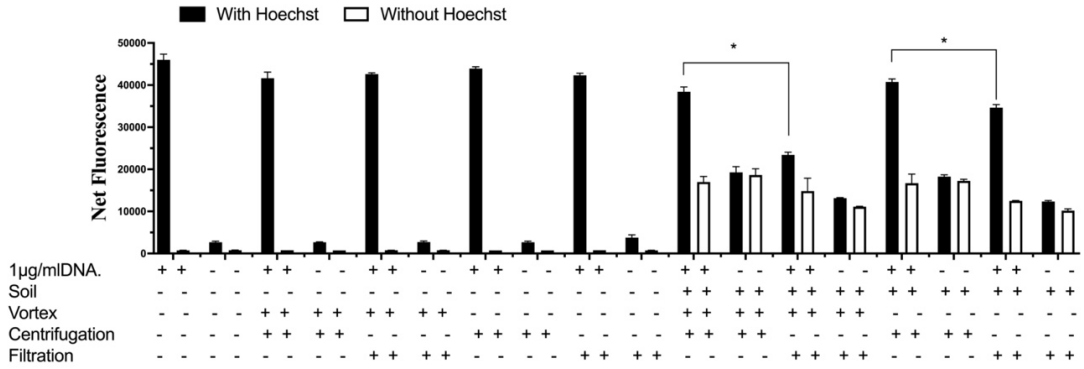


Figure 3.19. The impact of extraction method (centrifugation or filtration) on the extraction of DNA. Data represent average±SD (N=3). *Statistically significant by one-way ANOVA corrected with post-hoc Turkey test.

3.3.5 Optimisation of the spiking of DNA on the soil matrix

Having selected the best condition to reduce interferences, different approaches for spiking DNA on the soil were explored. DNA solution was prepared in TE buffer and spiked equally on soil and evaporated using an incubator at temperatures set at 30℃ and 60℃ (**Figure 3.20**). Both temperatures resulting in effective drying (spiking) of the DNA on the soil, with the lower temperature yielding higher signal.

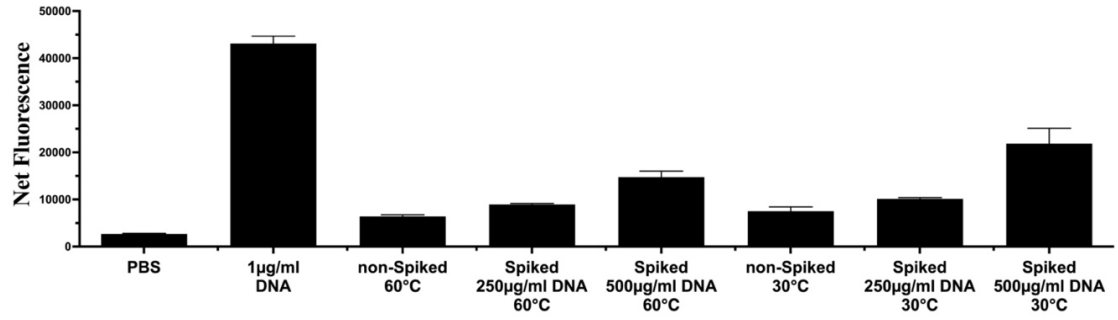


Figure 3.20. The method of spiking DNA on soil. Data represent average±SD (N=3).

To conclusively validate the detected fluorescence signal to DNA, an approach was selected to fragment DNA as a confirmation (**Figure 3.21**). The DNA spiked soil was extracted in PBS (3 ml) and then sonicated for 4 hours, which was enough to fragment DNA. Sonication resulted in a significant decrease of the signal, thus, confirming that the fluorescence signal is coming from the DNA on the spiked soil.

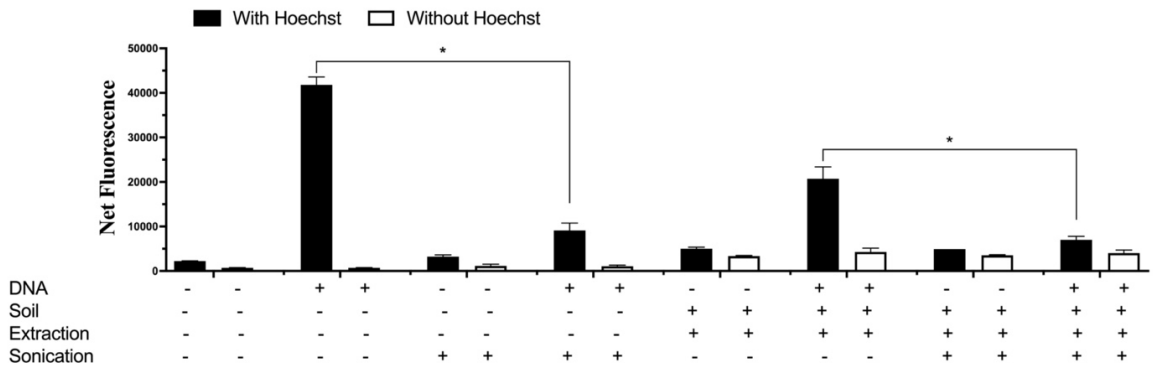


Figure 3.21. The impact of sonication on DNA extracted from spiked soil. Data represent average±SD (N=3). *Statistically significant by one-way ANOVA corrected with post-hoc Turkey test.

Finally, the recovery of the DNA from the spiked soil was explored. DNA-spiked soil simulants were extracted in 1ml PBS and then compared with the same concentration of pure DNA solution (**Figure 3.22**). 10 μg/ml DNA stock was made in TE buffer and then 20 μl was spiked on 50 mg JSC soil simulant. 30℃ overnight dry and then extracted in 1 ml PBS. The fluorescence of the extract was detected by Hoechst and the result showed the recovery of spiked-DNA can be around 12% (**Figure 3.22**). Different clays (Kaolinite (KGa-1b), Na-rich Montmorillonite (SWy-3), Montmorillonite (STx-1b) and Illite-smectite mixed layer (ISCz-1)) were also introduced in this experiment to explore the recovery but the initial test resulted in no detectable fluorescence from the Hoechst dye when introduced to these clays, indicating a lack of observable DNA recovery (**Figure 3.23**). Thus an auxiliary experiment was designed to check the specific influence of clay extracts on DNA, aiming to dissect whether the clays actively impeded DNA detection or interfered with the effectiveness of Hoechst dye.

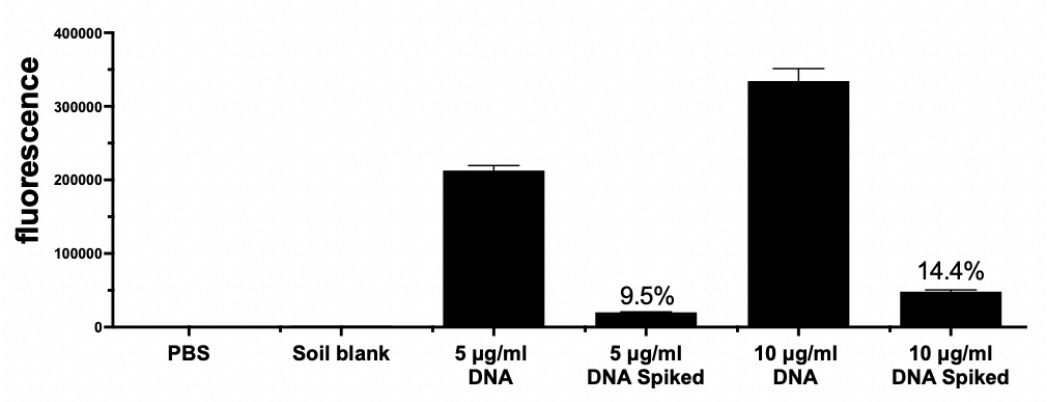


Figure 3.22. The recovery of DNA from spiked JSC soil simulants. Data represent average±SD (N=3).

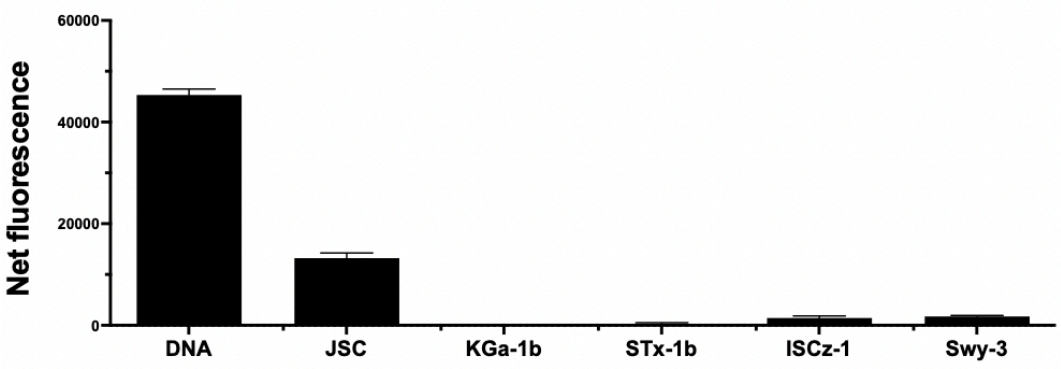


Figure 3.23. The recovery of DNA from spiked clay simulants. Data represent average±SD (N=3).

DNA stock was prepared in TE buffer and then diluted using JSC and clays extract (the control was DNA diluted in PBS). After incubation for 30 minutes, the fluorescence of each solution was measured by Hoechst (**Figure 3.24a**). Compared with the DNA in PBS, the extract of STx-1b, ISCz-1 and Swy-3 showed a significant decrease of the fluorescence signal which meant that DNA was degraded in these extracts. In contrast, the extract of KGa-1b did not lead to DNA destruction. However, upon spiking DNA onto it, fluorescence dyes failed to detect the DNA, which necessitating further investigation. In order to validate the degradation and visualize the existence of DNA, all these samples were run in electrophoresis (**Figure 3.24b**). From the image of electrophoresis, it confirmed the result of Hoechst fluorescence experiment.

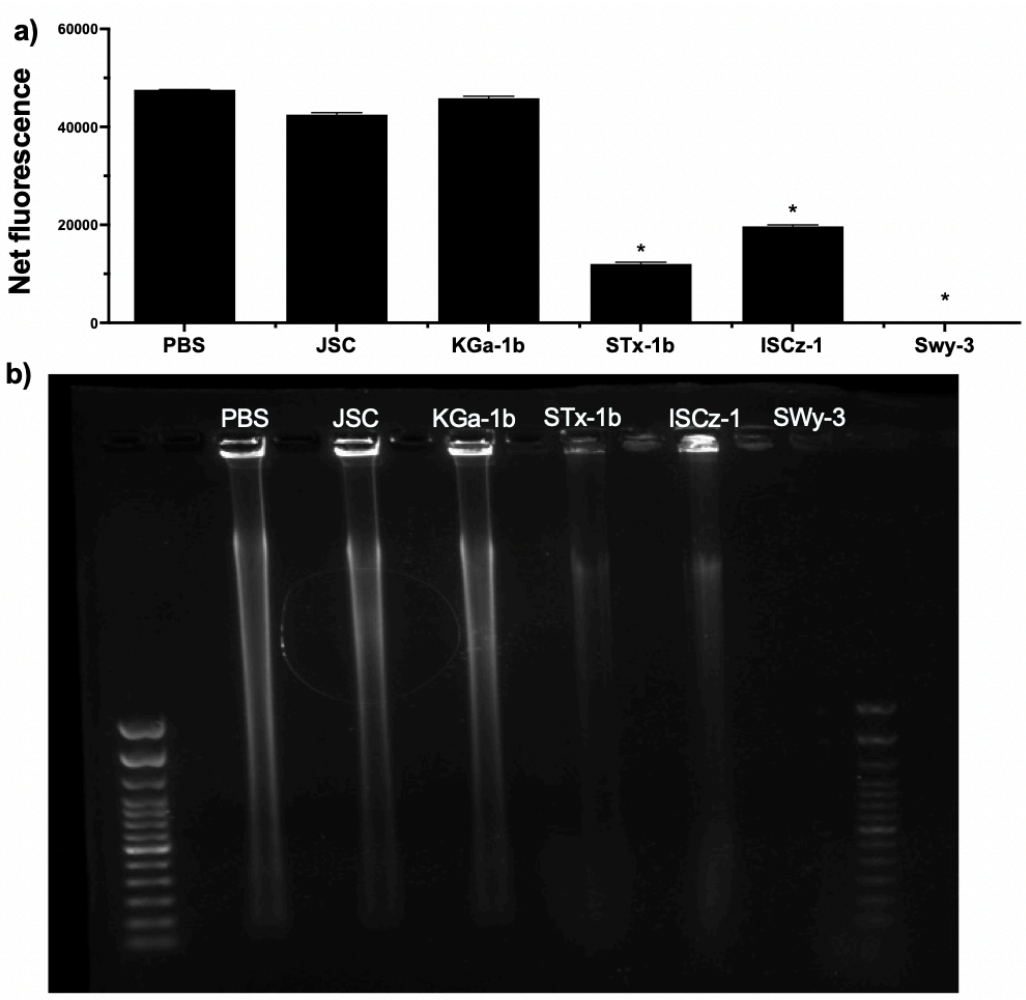


Figure 3.24. The impact of the clays extracts on DNA. Data represent average±SD (N=3). *Statistically significant by one-way ANOVA corrected with post-hoc Turkey test.

Since this method meets the criteria for a potential approach as a planetary exploration kit-based approach, the stability of the Hoechst fluorescent dye during storage was explored over 8 months at -20°C. The Hoechst dye was mixed with TE buffer, DMSO or PBS, separately, and defrosted periodically and compared for its sensitivity with the fresh Hoechst reagent **(Figure 3.25)**. The dye proved to be stable at -20°C, especially in DMSO, and retained its ability to detect and quantify DNA in soil simulants for the period of 8 months, which could indicate that this reagent could be prepared in a kit-based approach and sent in a future mission to assay DNA extracted samples in situ without compromising its sensitivity.

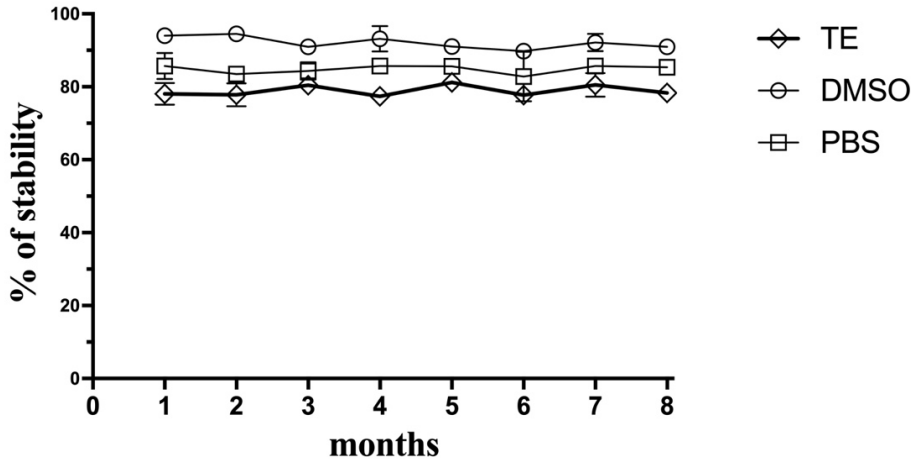


Figure 3.25. Stability of the Hoechst reagent over storage time. Hoechst was stored as (A) aliquots of the Hoechst reagent mixed with TE buffer frozen, (B) aliquots of the Hoechst reagent mixed with DMSO frozen, (C) aliquots of the Hoechst reagent mixed with PBS buffer frozen. Data represent average±SD (N=3).

3.3.6 The impact of UV radiation on DNA spiked on soil

Just as we observed in the prior chapter, UV radiation can pose substantial challenges to biological molecules, not limited to proteins but also extending to the realm of nucleic acids. The data accrued by the Curiosity rover on Mars indicates a total UV radiation dosage at Gale Crater of 20 W/m^2, or 72 kJ/hm^2 (Carrier et al., 2019). This measurement has been integral to the establishment of our experimental framework and has served as the foundation for our Martian UV radiation simulation. In the scope of the current chapter, we will be extrapolating our previous methodology to the investigation of DNA molecules. Herein, we will implement a similar strategy where DNA molecules will be integrated into soil simulants, then subjected to UV radiation approximating the Martian dosage of 72 kJ/hm^2. This replication of Martian conditions facilitates an in-depth examination of the possible effects of UV radiation on DNA integrity, offering a more holistic perspective on the potential survival or degradation of biological molecules on Mars. Soil (50 mg JSC Mars-1A Martian Regolith Simulant) was incubated with 50 µl 500 µg/ml DNA solution in TE buffer and then dried in the incubator at 30 ℃ for 24 hours. DNA were suspected remain into soils and then spiked

soils were exposed under UV light for 3 hours. Following, they were extracted in PBS and quantified by the Hoechst (**Figure 3.26**).

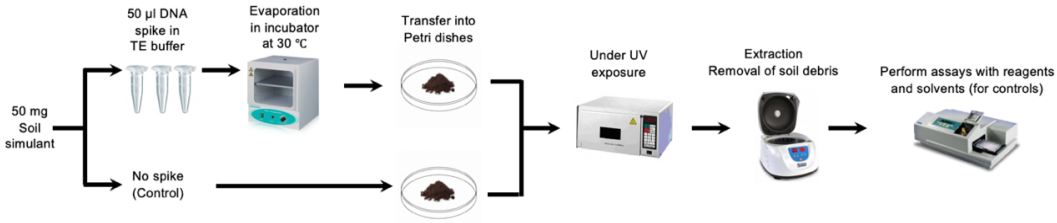


Figure 3.26. Spiking methods of soil samples with DNA under UV radiation.

To ensure the accuracy of the experiment and additionally provide evidence that the UV has the potential to fragment DNA, controls were prepared without UV spiked soils but also with pure DNA (**Figure 3.27**).

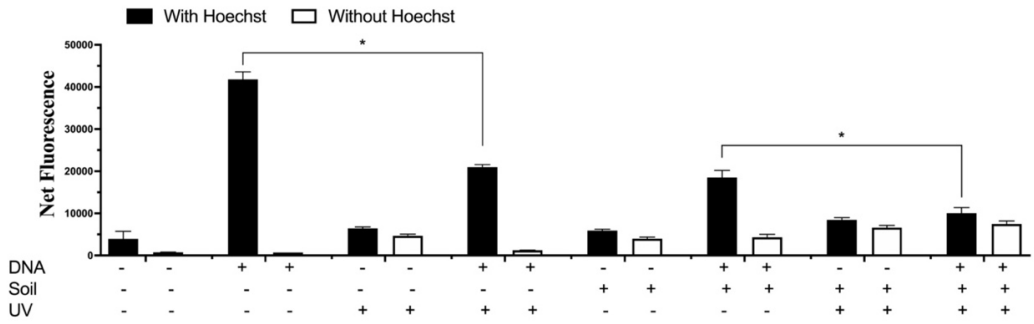


Figure 3.27. The impact of UV radiation on DNA spiked soil. Data represent average±SD (N=3). *Statistically significant by one-way ANOVA corrected with post-hoc Turkey test.

3.3.7 The impact of temperature on DNA spiked on soil

In continuity with the preceding chapter, this study further extends its investigation into the thermal stability of biological molecules, with the current focus shifting towards DNA. Mars is characterized by a thin atmosphere which scarcely retains heat, causing drastic temperature disparities between day and night (Fairén, 2010). The average Martian temperature hovers around negative 60 degrees Celsius. Equatorial regions on Mars can reach 20 degrees Celsius during a summer day, but at night, these temperatures can plummet to negative 73 degrees Celsius. During winter, the poles can average negative 80 degrees Celsius, with the temperature occasionally dropping to a

chilling negative 125 degrees Celsius (Leovy, 2001).

In this chapter, we adopted a similar approach to the preceding chapter by examining the stability of DNA both in isolation and when spiked into soil samples, under the severe cold temperatures that characterize the Martian environment, particularly at negative 18 degrees Celsius and negative 80 degrees Celsius (**Figure 3.28**). By emulating the extreme temperature conditions varying from the equatorial region to the poles of Mars, this segment provides a comprehensive evaluation of the potential effects of Martian cold on DNA stability. This replication of the Martian environment, in combination with the UV radiation exposure experiments, provides us with a more realistic understanding of the potential degradation or survival of DNA molecules on Mars. The results indicated that DNA and the DNA-spiked soil samples exhibited significant stability under both -18 and -80-degree conditions. The resilience of DNA under such extreme cold contributes substantially to our understanding of the potential for the preservation of biological signatures in the Martian environment.

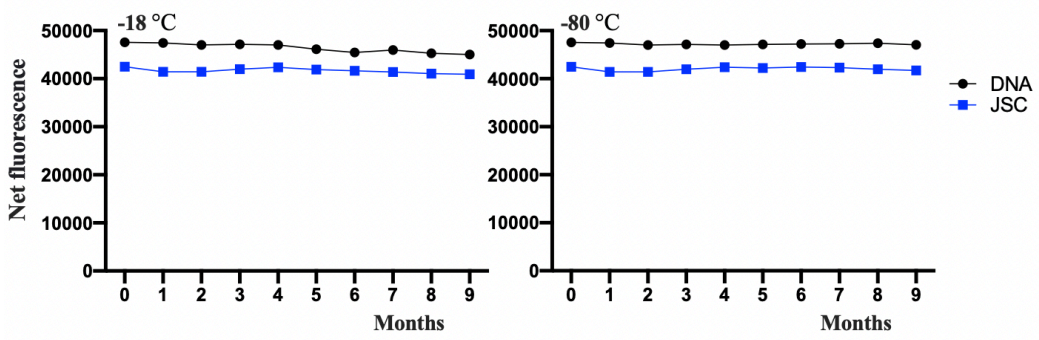
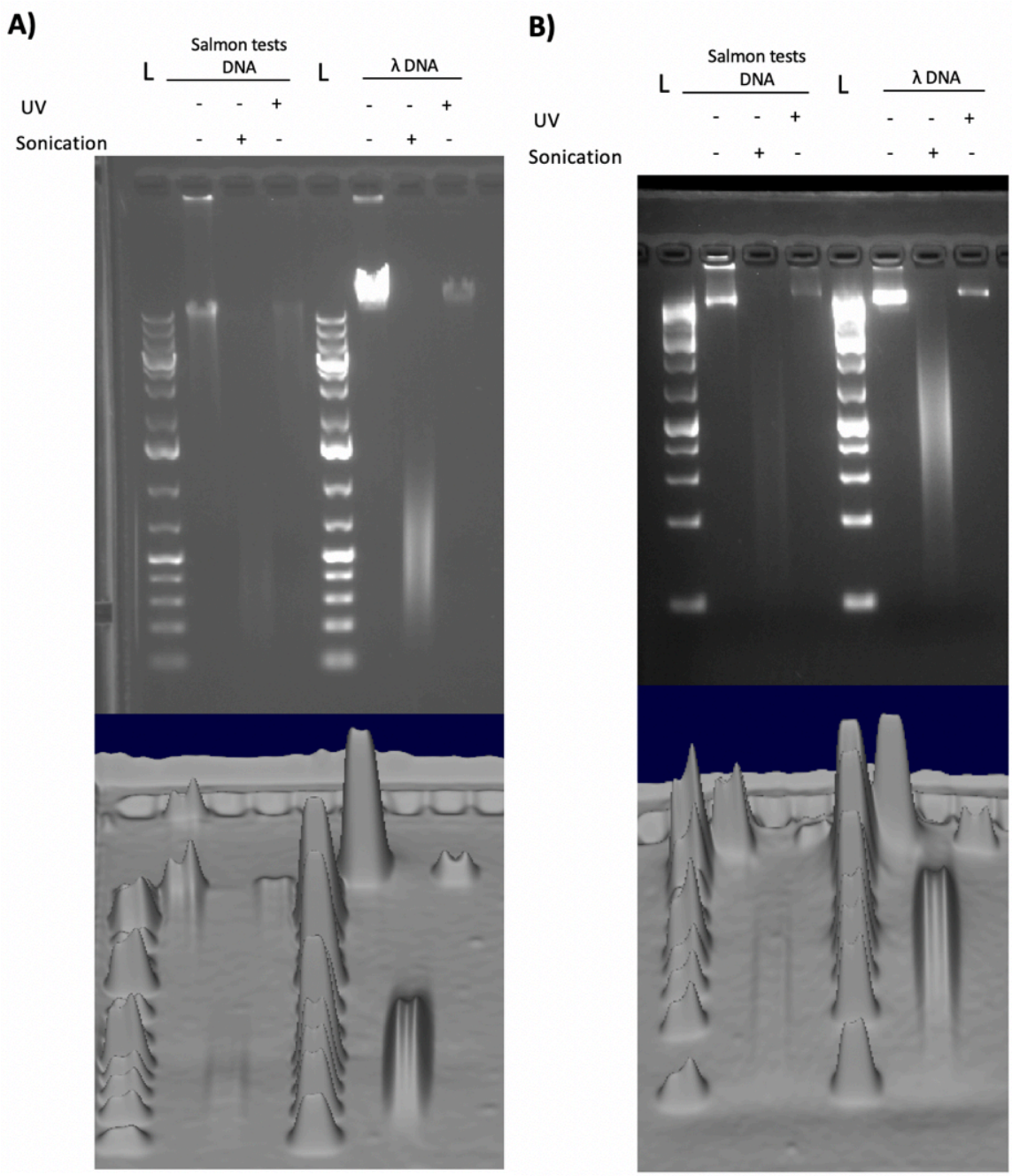


Figure 3.28. Thermal stability of DNA in extreme cold conditions. The figure presents the resilience of both isolated DNA and DNA spiked-soil samples under temperatures of -18°C and -80°C, representative of Mars' harsh cold climate. Data represent average±SD (N=3).

3.3.8 Validation of the spiking of DNA on the soil matrix

In order to validate the results of the spiking of DNA on the soil matrix, the electrophoresis was introduced to ensure and visualize the existence of DNA. First of all, two different DNA stocks (Salmon test DNA and λ DNA) were prepared and

executed with UV radiation or sonication separately. Then, the electrophoresis was used to show the existence of DNA, to ensure the validity of the result, the DNA samples were also quantified by Hoechst (**Figure 3.29**). From the image of the electrophoresis, DNA samples treated by sonication showed a smear band while DNA samples treated by UV radiation showed a dimly band. This result indicated that sonication could break DNA molecules into small pieces while UV radiation could make damage to DNA molecules.


A)
L
Salmon tests DNA
L
λ DNA
UV
- - + - - +
Sonication
- + - - + -
B)
L
Salmon tests DNA
L
λ DNA
UV
- - + - - +
Sonication
- + - - + -

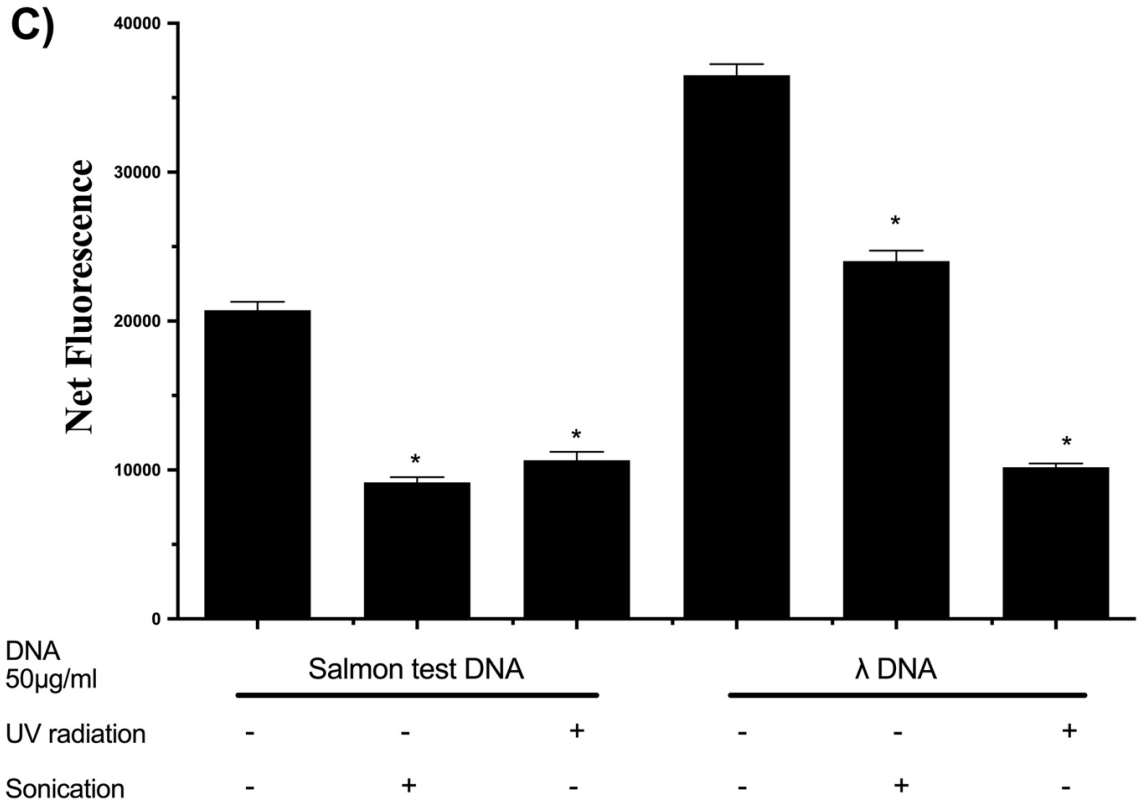
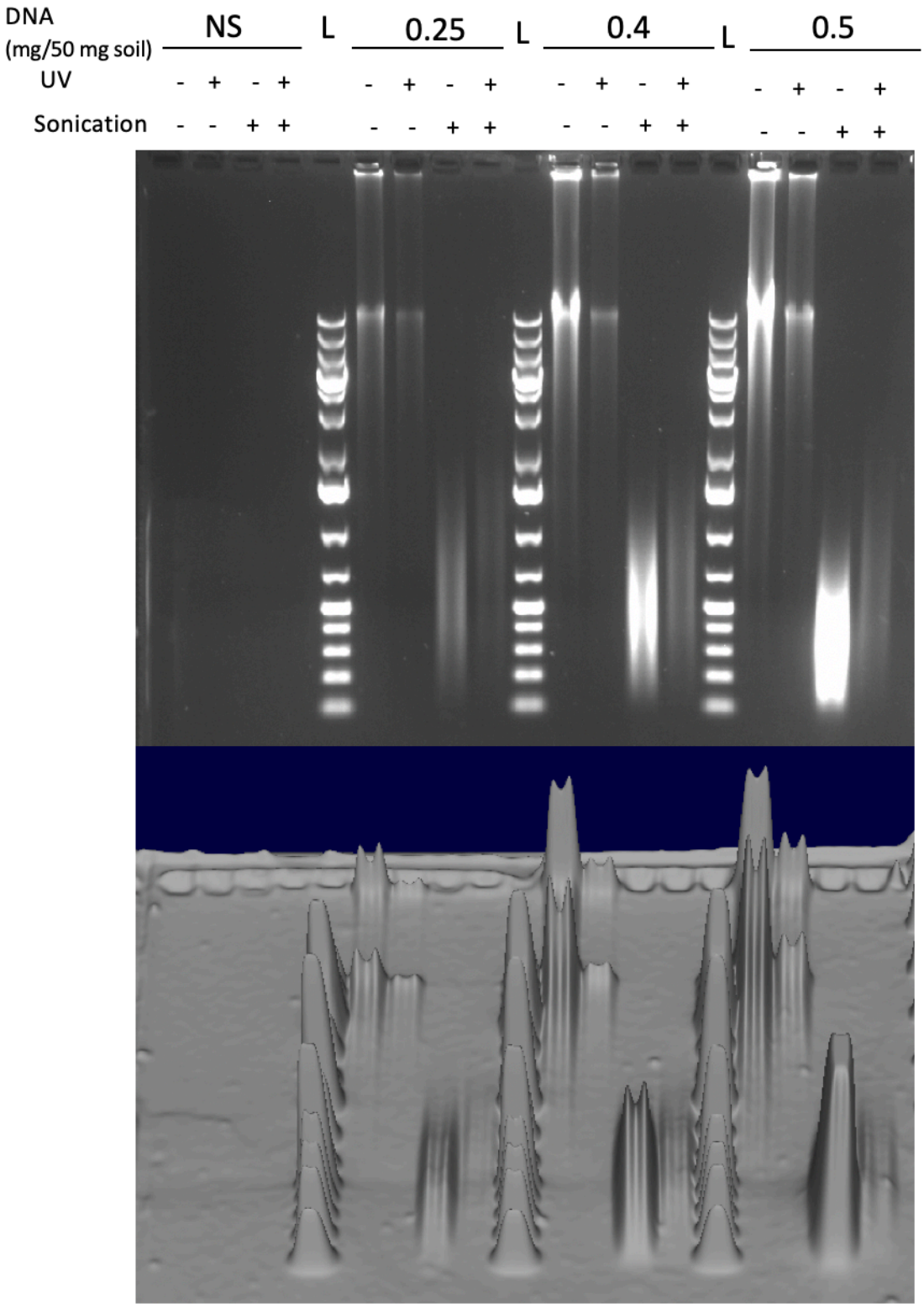


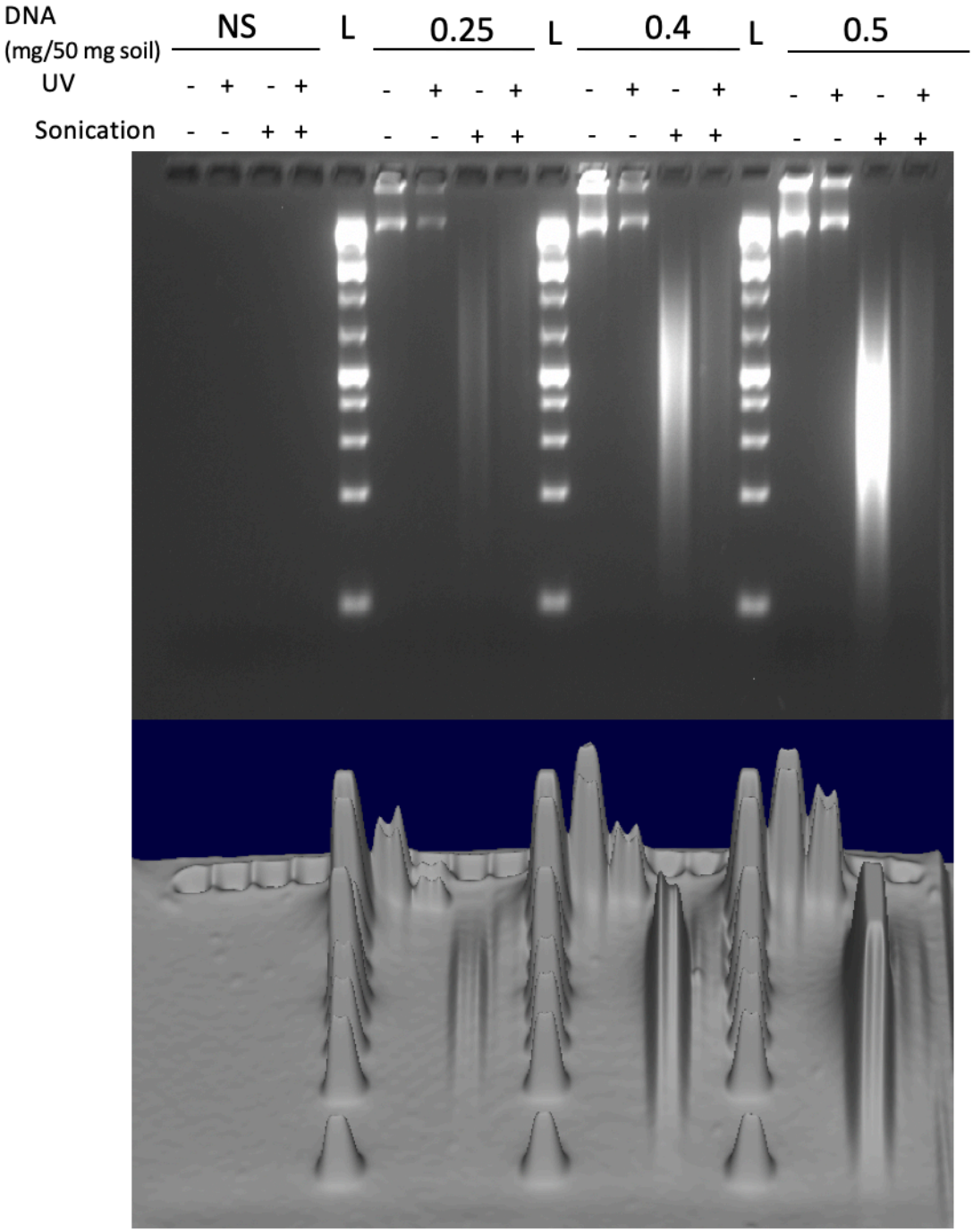
Figure 3.29. Electrophoretic analysis and Hoechst results for DNA in solution. Electrophoresis at 1% (A) or 3% (B) agarose and quantification of the DNA by Hoechst (C). Data represent average±SD (N=3). *Statistically significant by one-way ANOVA corrected with post-hoc Turkey test.

Following, salmon testes DNA was spiked on the soil matrix and executed with UV radiation. Then the spiked soil matrix (with or without UV radiation) was extracted in PBS, the extraction was executed with sonication. These samples were both analysed by electrophoresis and quantified by the Hoechst dye. Non-spiked soil matrix was used as a control (**Figure 3.30**). From the image of the electrophoresis, there were no bands for the non-spiked soil matrix which provided a clear control to ensure the following detected DNA signal was all from spiked soil matrix. The result was similar to the previous experiment which used pure DNA stock. Sonication could break DNA molecules into small pieces while UV radiation could make damage to DNA molecules.

A)



B)



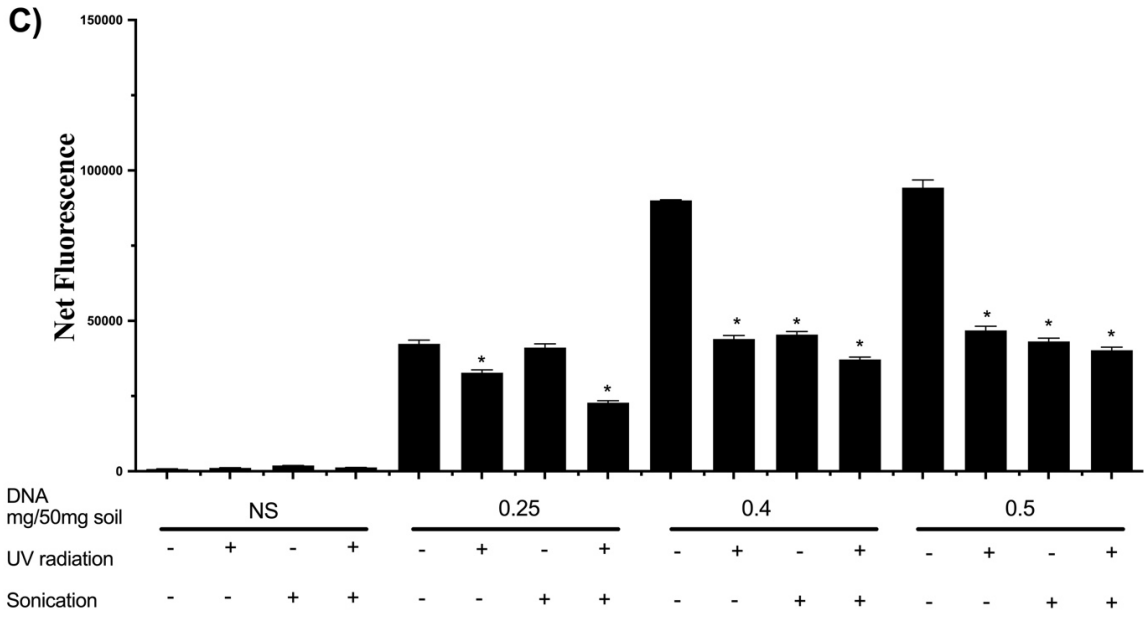
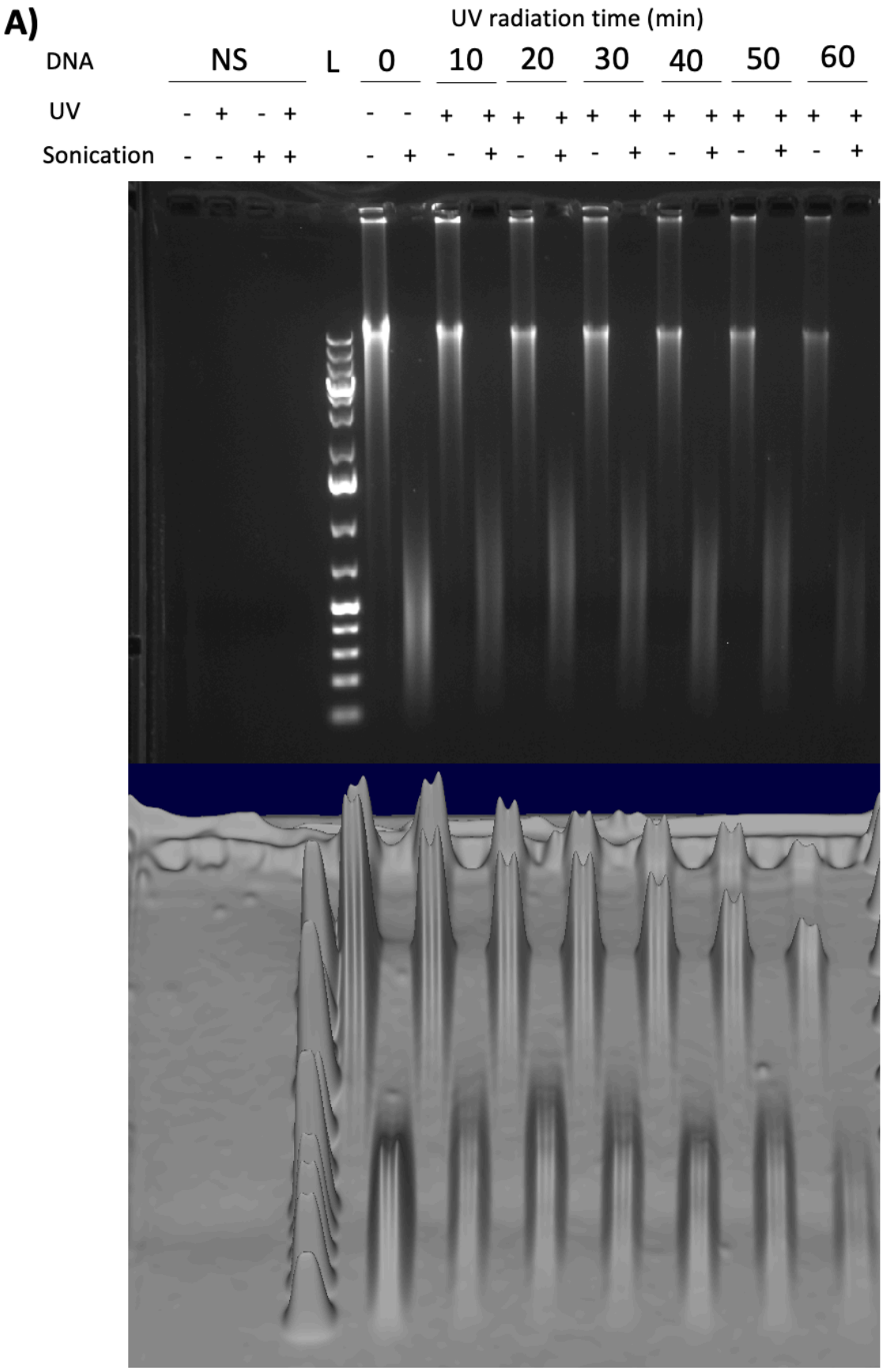
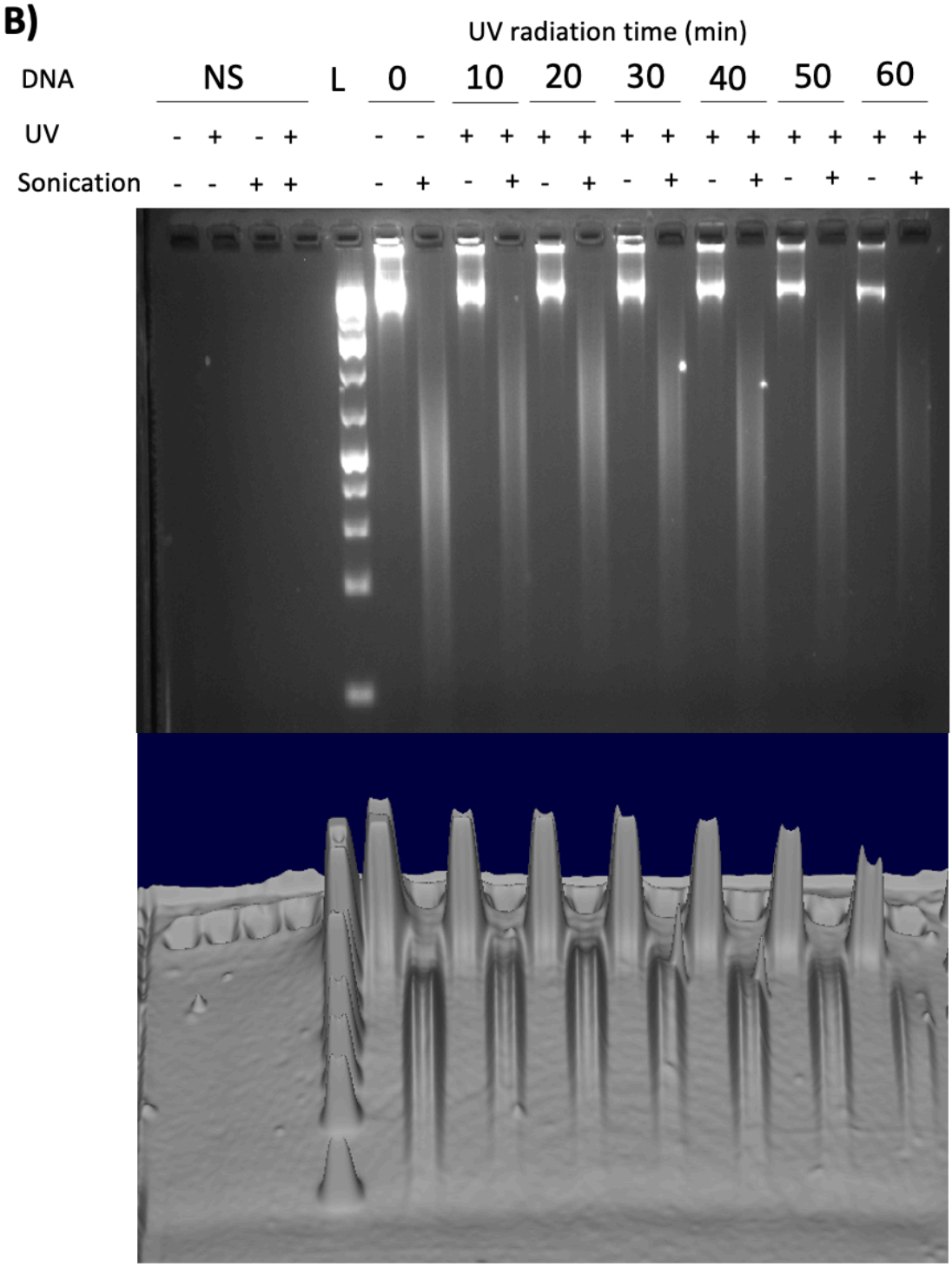


Figure 3.30. Electrophoretic analysis and Hoechst results for DNA in spiked soil matrix. Electrophoresis at 1% (A) or 3% (B) agarose and quantification of the DNA by Hoechst (C). Data represent average±SD (N=3). *Statistically significant by one-way ANOVA corrected with post-hoc Turkey test.

In order to explore the impact of UV radiation time on the DNA-spiked soil matrix, the spiked soil matrix was exposed under different UV radiation time points. Then the spiked soil matrix was extracted in PBS, the extraction was executed with sonication. These samples were both analysed by electrophoresis and quantified by the Hoechst dye. Non-spiked soil matrix was used as a control (**Figure 3.31**). From the image of the electrophoresis, there were no bands for the non-spiked soil matrix which provided a clear control to ensure the following detected DNA signal was all from spiked soil matrix. The result showed that with the increase of UV radiation time, DNA was gradually degraded.


A)
UV radiation time (min)
DNA
NS
L
0
10
20
30
40
50
60
UV
- + - + - - + + + + + + + + + + + +
Sonication
- - + + - + - + - + - + - + - + - +

B)



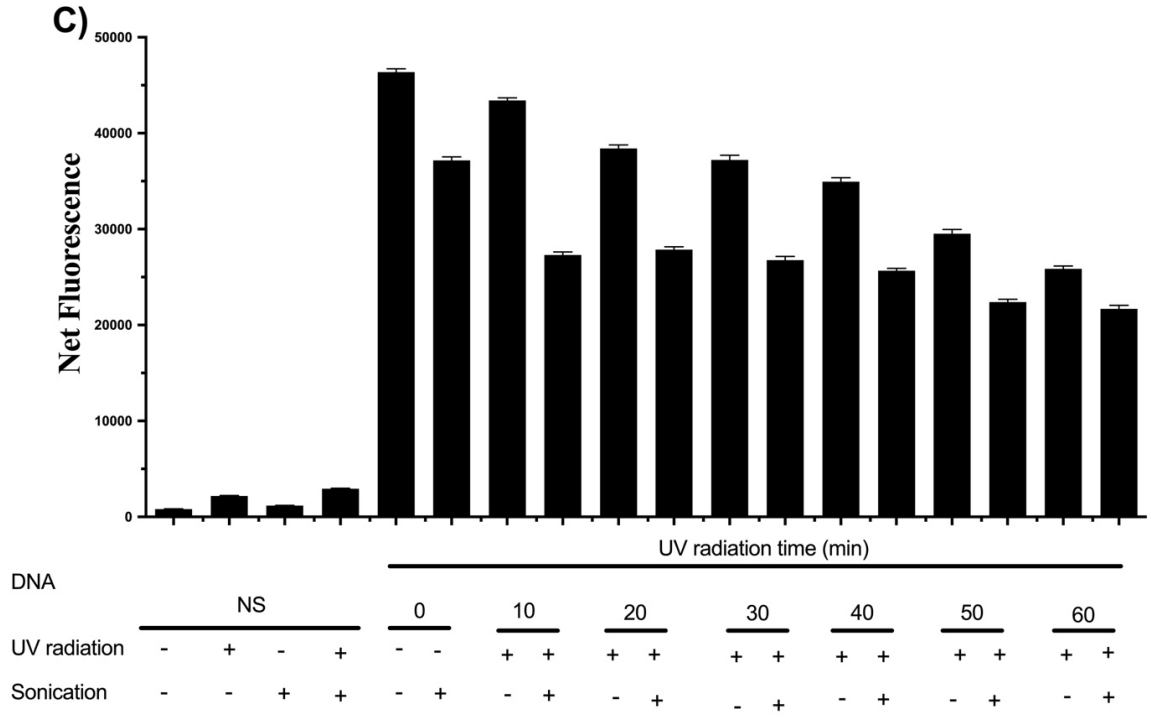
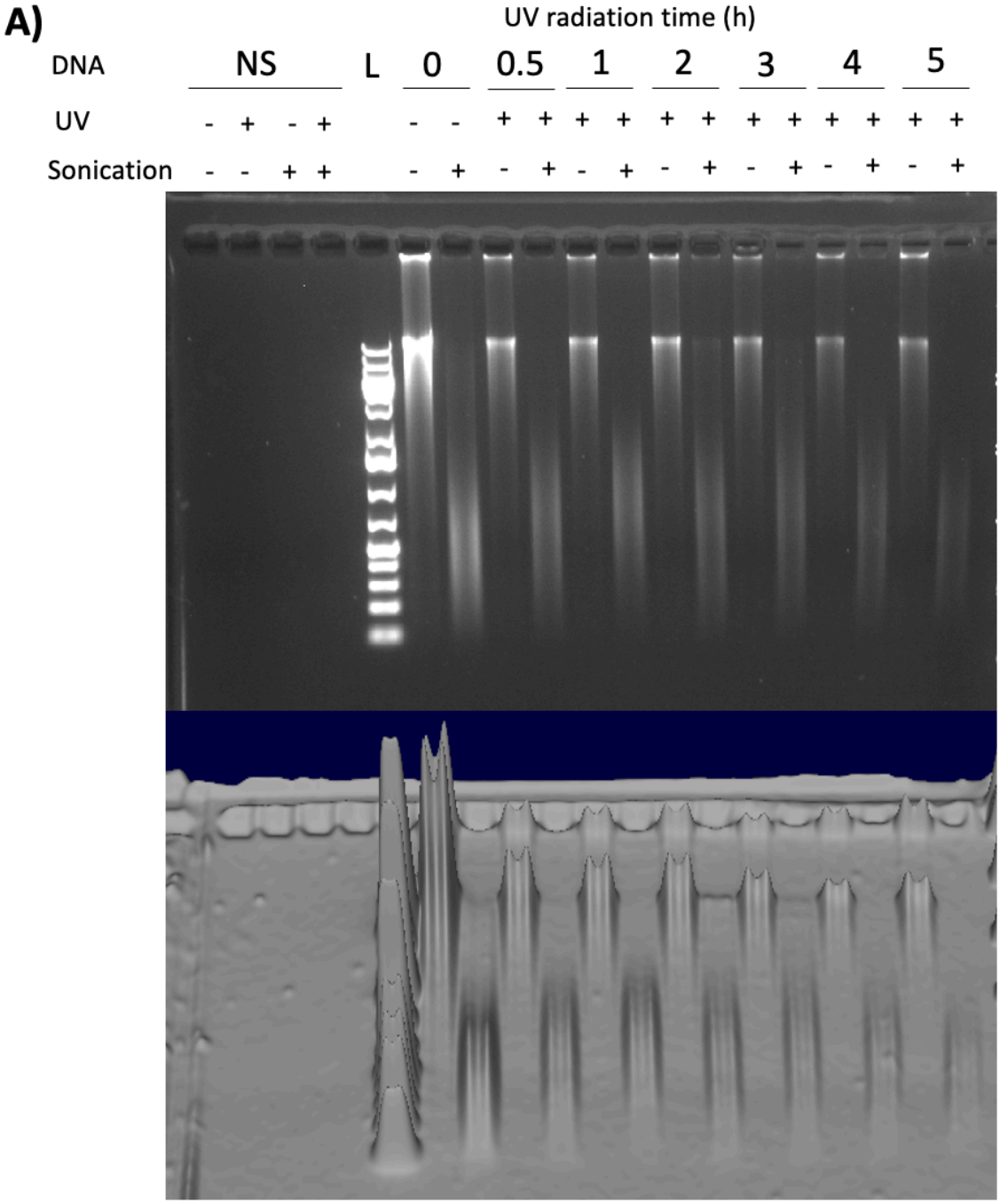


Figure 3.31. Electrophoretic analysis and Hoechst results for the impact of UV radiation on DNA in spiked soil matrix. Electrophoresis at 1% (A) or 3% (B) agarose and quantification of the DNA by Hoechst (C). Data represent average±SD (N=3).

When the UV radiation time increased longer, the trend of the DNA concentration had leveled out (**Figure 3.32**).


A)
UV radiation time (h)
DNA
NS
L
0
0.5
1
2
3
4
5
UV
- + - + - - + + + + + + + + + + + +
Sonication
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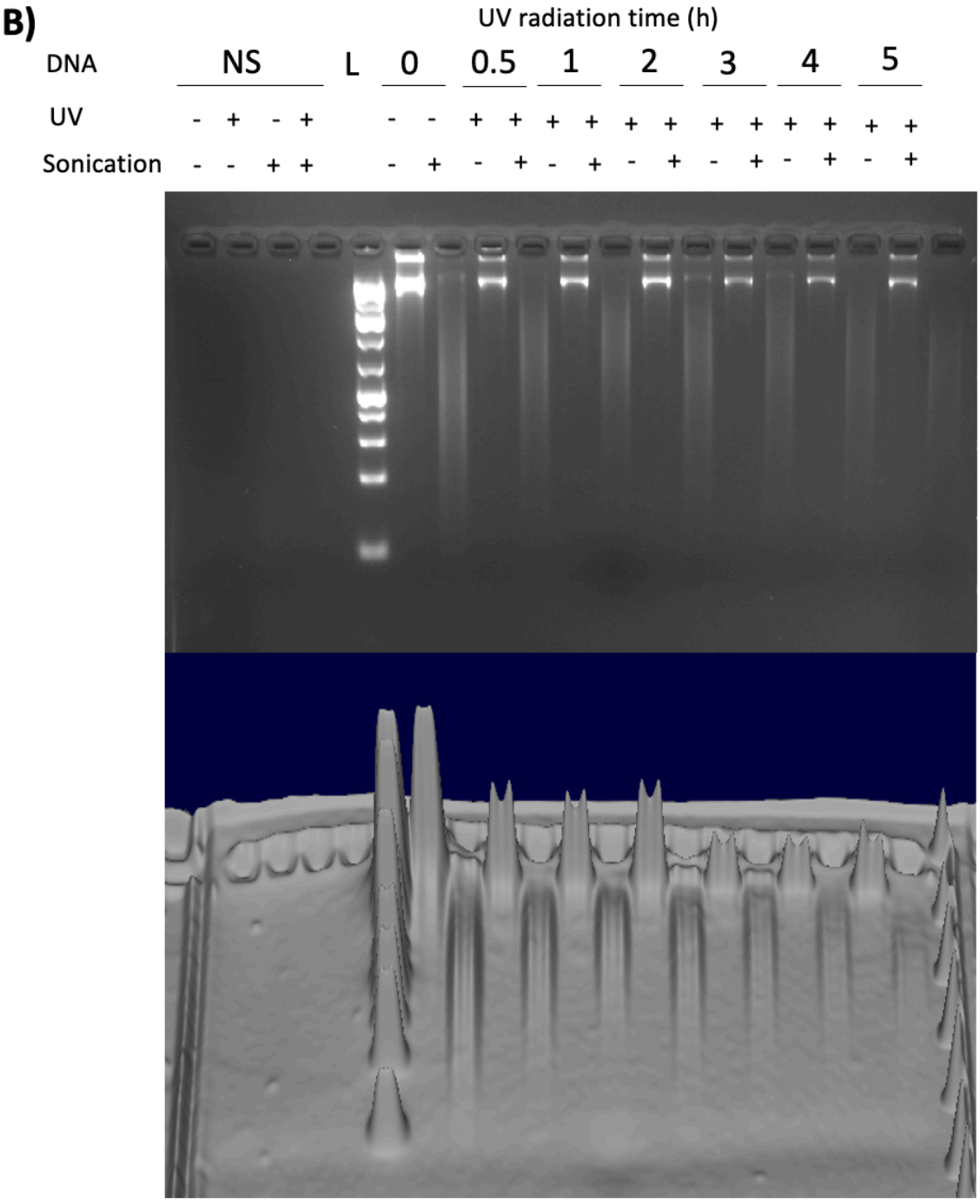
B)



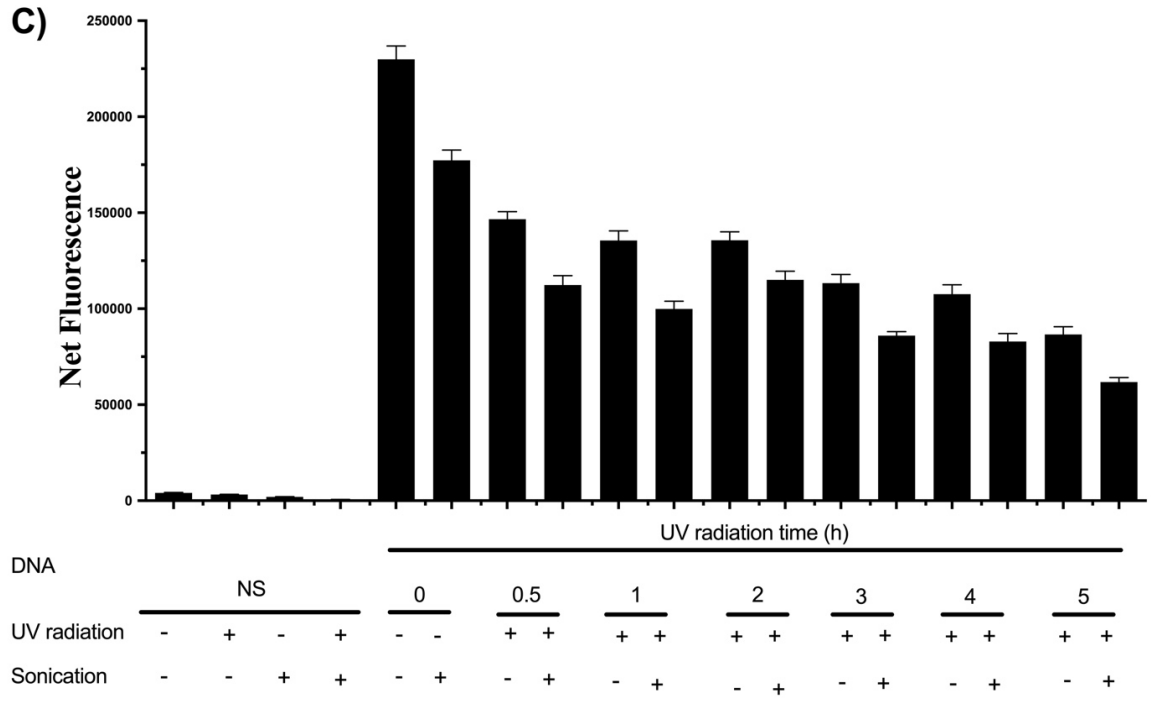


Figure 3.32. Electrophoretic analysis and Hoechst results for the impact of UV radiation (up to 5 hours) on DNA in spiked soil matrix. Electrophoresis at 1% (A) or 3% (B) agarose and quantification of the DNA by Hoechst (C). Data represent average±SD (N=3).

3.4 Discussion

While the Introduction provided a general background on the quest for detecting DNA on extra-terrestrial terrains, in this Discussion, we delve into the specific implications, limitations, and future directions of our study. In order to detect nucleic acids, using fluorescent dyes is considered a quick approach with high sensitivity and selectivity. Catalysed hairpin assembly (CHA) is a novel technology to amplify fluorescent signals based on two partially complementary hairpins (Zou et al., 2021). For example, thiazole orange (TO) is a cyanine dye and exhibits strong fluorescence when it combines with double-stranded DNA. In the research of Zou et al. (2021), TO was embedded in two molecule beacons (MBs) to detect H5N1 DNA with the presence of molybdenum disulfide (MoS_2) which can lower the background signal duo to its high fluorescence quenching ability. This assay can be applied in the clinical for virus DNA detection with advantage of low-cost and low-background signal. However, this assay required the preparation of MoS_2 nanosheets by using chemical exfoliation method (Li et al., 2015) and the reaction needed to incubate at 37 °C for one hour to the fluorescence measurement. Compared to our assay, all three fluorescent dyes that we used were only need to 10 minutes incubation and were ready to measure the fluorescence. It is much quicker and there was no need to prepare extra chemicals to reduce the background signal. On the other hand, the MoS_2-based TO assay only showed the high sensitivity and specificity for H5N1 DNA, for the detection of other DNA, it needed to change the corresponding molecular beacons to achieve the different DNA detection. This is useful to the specific DNA detection, while for a planetary mission, the potential DNA type will be unknown and complicated. The assay developed can detect different types of DNA with the high sensitivity, and the electrophoresis was also introduced to ensure and visualize the existence of DNA.

Besides fluorescent methods for the detection of DNA, PCR (polymerase chain reaction)-based technology is another popular DNA detection and genotyping technique. In the study of Wang et al. (2018), a novel DNA detection method based on the Cas9 nuclease was described. This method included two steps of PCR based on the

CRISPR/Cas9 system to detect and type target DNA rapidly and specifically (Wang et al., 2018). However, duo to the difficulty of multiple amplification of PCR technique and the challenge of the design of specific primers, it is limited to detect specific DNA type. In addition, such techniques require more steps (thermal PCR cycles), a number of reagents which are prone to stability and may not be the most suitable approach especially for an *in situ* planetary exploration method.

Another promising approach is sequencing DNA. So far, many technologies already developed in laboratory stage to detect and sequence DNA, have been possible to be scaled down in instrumentation to fit on a rover, are able to survive transport from Earth to space, and conduct high fidelity sequencing in the harsh and complicated environment on Mars is still a unique challenge.

As the existence of the extreme environment on the surface of Mars, such as subzero temperature, strong UV radiation, dryness, harsh oxidizing conditions and a thin carbon-rich atmosphere (95% CO_2 occupied), it resulted that on the surface of Mars, the abundance of life could be very low (Direito et al., 2012). In this research, our primary focus lies on the interplay between the impact of temperature conditions and UV radiation exposure on DNA, as these two environmental factors play significant roles in the Martian environment. The findings of this study demonstrate a temporal correlation between the duration of ultraviolet (UV) radiation exposure and a decline in the fluorescent signal corresponding to DNA concentration. This trend was established through comparative analysis with sonication-treated samples. Gel electrophoresis further corroborated these observations. Specifically, DNA samples exposed to sonication yielded a diffused band pattern on the agarose gel. In contrast, UV-treated samples manifested a discernible reduction in band intensity, thereby supporting the hypothesis that the concentration of the targeted DNA fragment diminishes upon UV exposure. This result was also proved by Hansen et al. (2009), which found that cells of cyanobacteria and *Bacillus* strains can only survive for 3 hours maximumly under the exposure to the UV radiation. However, by analysing different simulated Martian minerals under the exposure of UV radiation, it showed different

minerals had different degrading effect on DNA, especially on nucleotides (Fornaro et al., 2018). For instance, labradorite and natrolite can promote the degrading process of nucleotides, whereas apatite, lizardite and antigorite did not have the degrading effect on nucleotides (Fornaro et al., 2018). In addition to the effects of UV radiation on DNA integrity, it is noteworthy to consider the potential role of clays in preserving organic compounds. Clays possess unique structural characteristics that allow them to harbour organic moieties within their interlayer spaces (Chiu et al., 2014). The lattice-like structure of these minerals creates a protective microenvironment that can shield organic molecules from external degradation factors, including but not limited to, UV radiation, oxidative stress, and extreme temperatures. This sequestration phenomenon could significantly impact the bioavailability and longevity of DNA molecules under harsh environmental conditions, offering a natural avenue for the preservation of genetic material. Based on this, the absence of detectable fluorescence signals in the extract of KGa-1b could be attributed to the intricate, lattice-like structural composition of the clay, rather than to the degradation of DNA. It is plausible that DNA molecules are sequestered within these complex structural frameworks, rendering them inaccessible to standard extraction protocols and consequently, undetectable by fluorescence assays. This scenario underscores the potential limitations of conventional extraction techniques in recovering DNA from complex mineral matrices and highlights the need for advanced methodologies to enhance the efficiency and effectiveness of DNA retrieval and detection in such contexts. Concurrently, the investigation into the temperature-related stability of DNA and DNA-spiked soil samples under Martian conditions (-18 to -80 degrees Celsius) provided valuable insights. It indicated that soil layers provide limited protection against direct UV radiation, whereas temperature conditions provide effective protection. This finding contributed the choice of the landing site on Mars where could provide the potential life samples. Nonetheless, it's crucial to note that these are simplified laboratory conditions, which, while replicating some key features of the Martian environment, do not fully capture its complexity. The actual Martian environment may present additional challenges such as desiccation, oxidation, and other physical and chemical interactions with Martian soil and

atmospheric elements, which may pose further obstacles to DNA preservation and detection. This, however, does not undermine the significance of our findings, but rather points out the areas where further research and development are needed to prepare for the stringent demands of extra-terrestrial exploration.

For the Mars exploration, to develop an *in situ* life detection instrument is crucial to search the evidence of life on Mars. Fornaro's study suggested the best place to land and drill, while in the study of Mojarro et al. (2019), it developed a metagenomics-based life-detection instrument which combined the technology of nucleic acid extraction and nanopore sequencing. This instrument can isolate nucleic acid from soils on Mars and sequence it to compare with the life on Earth (Mojarro et al., 2019). However, this technique can be only applied to the extant or recently dead cells because during the process for preparing samples, the DNA preserved in soil could be destroyed and cannot be detected. According to our fluorescent assays, it is sensitive to the different type of DNA, and the process for preparing samples is friendly to DNA, which will not destroy the DNA preserved in soil. This optimized fluorescent assays could be adapted to the Mojarro's instrument for the DNA detection at the first attempt.

3.5 Conclusion

In this chapter, we refined a biochemical method geared towards the sensitive, simple, and efficient detection of DNA, with a central focus on the development of fluorescent assays and DNA quantification in simulated Martian soil. The troubleshooting for this method addressed the critical facets of reaction stability, the interrelation between measurement gain, sensitivity, and background fluorescence. The resultant optimized method proposed using PBS for soil simulant spiking, followed by a delicate evaporation process.

To confirm the effectiveness of our method, we conducted electrophoretic analyses. We examined the influence of various environmental stressors, including UV radiation, low temperature, and sonication, on soil samples and we highlighted the resilience and retention of DNA within soil matrices. The pronounced degradation effects underlined by these conditions serve as a testament to the rigors the DNA undergoes.

The steps and modifications detailed throughout this chapter offer a streamlined, cost-effective, and adept protocol for DNA detection in simulated Martian soils. While our findings contribute a significant piece to the complex puzzle of astrobiological research, we acknowledge that it is one of many steps in the broader and evolving journey to ascertain the possibilities of extra-terrestrial life.

3.6 Reference

BEAUCHAMP, P. & BELZ, A. 2013. Assessing planetary protection and contamination control technologies for planetary science missions. *2013 IEEE Aerospace Conference.*

BOWATER, R. P. & BRAZDA, V. 2022. Impacts of Molecular Structure on Nucleic Acid-Protein Interactions. *Int J Mol Sci,* 24.

BROWN, D. & LANDAU, E. 2019. *NASA-Funded Research Creates DNA-like Molecule to Aid Search for Alien Life* [Online]. Available: https://www.nasa.gov/press-release/nasa-funded-research-creates-dna-like-molecule-to-aid-search-for-alien-life [Accessed August 23rd 2021].

CALLAHAN, M. P., SMITH, K. E., CLEAVES, H. J., 2ND, RUZICKA, J., STERN, J. C., GLAVIN, D. P., HOUSE, C. H. & DWORKIN, J. P. 2011. Carbonaceous meteorites contain a wide range of extraterrestrial nucleobases. *Proc Natl Acad Sci U S A,* 108**,** 13995-8.

CARRIER, B. L., ABBEY, W. J., BEEGLE, L. W., BHARTIA, R. & LIU, Y. 2019. Attenuation of Ultraviolet Radiation in Rocks and Minerals: Implications for Mars Science. *Journal of Geophysical Research: Planets,* 124**,** 2599-2612.

CAVALAZZI, B. & WESTALL, F. 2019. *Biosignatures for Astrobiology*, Springer, Cham.

CHIU, C.-W., HUANG, T.-K., WANG, Y.-C., ALAMANI, B. G. & LIN, J.-J. 2014. Intercalation strategies in clay/polymer hybrids. *Progress in Polymer Science,* 39, 443-485.

CRICK, F. 1970. Central dogma of molecular biology. *Nature,* 227**,** 561-3.

DEMETS, R. 2012. Darwin's contribution to the development of the Panspermia theory. *Astrobiology,* 12**,** 946-50.

DEVINE, K. G. & JHEETA, S. 2020. De Novo Nucleic Acids: A Review of Synthetic Alternatives to DNA and RNA That Could Act as Bio-Information Storage Molecules. *Life (Basel),* 10.

DIREITO, S. O., MAREES, A. & ROLING, W. F. 2012. Sensitive life detection

strategies for low-biomass environments: optimizing extraction of nucleic acids adsorbing to terrestrial and Mars analogue minerals. *FEMS Microbiol Ecol,* 81**,** 111-23.

DRAGAN, A. I., CASAS-FINET, J. R., BISHOP, E. S., STROUSE, R. J., SCHENERMAN, M. A. & GEDDES, C. D. 2010. Characterization of PicoGreen interaction with dsDNA and the origin of its fluorescence enhancement upon binding. *Biophys J,* 99**,** 3010-9.

ENYA, K., YAMAGISHI, A., KOBAYASHI, K. & YOSHIMURA, Y. 2022. Comparative study of methods for detecting extraterrestrial life in exploration mission of Mars and the solar system. *Life Sci Space Res (Amst),* 34**,** 53-67.

EXPLORINGORIGINS. 2020. *THE RNA WORLD HYPOTHESIS* [Online]. Available: http://exploringorigins.org/ribozymes.html [Accessed May 5th 2020].

FAIRÉN, A. G. 2010. A cold and wet Mars. *Icarus,* 208**,** 165-175.

FORNARO, T., BOOSMAN, A., BRUCATO, J. R., TEN KATE, I. L., SILJESTRÖM, S., POGGIALI, G., STEELE, A. & HAZEN, R. M. 2018. UV irradiation of biomarkers adsorbed on minerals under Martian-like conditions: Hints for life detection on Mars. *Icarus,* 313**,** 38-60.

FORTERRE, P. 2001. Genomics and early cellular evolution. The origin of the DNA world. *Comptes Rendus de l'Académie des Sciences - Series III - Sciences de la Vie,* 324**,** 1067-1076.

FORTERRE, P., FILÉE, J. & MYLLYKALLIO, H. 2004. Origin and Evolution of DNA and DNA Replication Machineries. *The Genetic Code and the Origin of Life.*

GALLAGHER, S. R. & DESJARDINS, P. R. 2008. Quantitation of DNA and RNA with absorption and fluorescence spectroscopy. *Curr Protoc Protein Sci,* Appendix 3**,** Appendix 4K.

GARNER, D. L. 2009. Hoechst 33342: the dye that enabled differentiation of living X- and Y-chromosome bearing mammalian sperm. *Theriogenology,* 71**,** 11-21.

GEBERT, L. F. R. & MACRAE, I. J. 2019. Regulation of microRNA function in animals. *Nat Rev Mol Cell Biol,* 20**,** 21-37.

GLICK, B. R., PASTERNAK, J. J. & PATTEN, C. L. 2010. *Molecular biotechnology :*

principles and applications of recombinant DNA, ASM Press, Washington, DC.

GREEN, S. J., TOROK, T., ALLEN, J. E., ELOE-FADROSH, E., JACKSON, S. A., JIANG, S. C., LEVINE, S. S., LEVY, S., SCHRIML, L. M., THOMAS, W. K., WOOD, J. M. & TIGHE, S. W. 2023. Metagenomic Methods for Addressing NASA's Planetary Protection Policy Requirements on Future Missions: A Workshop Report. *Astrobiology*.

HANSEN, A. A., JENSEN, L. L., KRISTOFFERSEN, T., MIKKELSEN, K., MERRISON, J., FINSTER, K. W. & LOMSTEIN, B. A. 2009. Effects of long-term simulated martian conditions on a freeze-dried and homogenized bacterial permafrost community. *Astrobiology,* 9**,** 229-40.

HOLDEN, M. J., HAYNES, R. J., RABB, S. A., SATIJA, N., YANG, K. & BLASIC, J. R., JR. 2009. Factors affecting quantification of total DNA by UV spectroscopy and PicoGreen fluorescence. *J Agric Food Chem,* 57**,** 7221-6.

IRWIN, L. N. & SCHULZE-MAKUCH, D. 2011. *Cosmic Biology*.

JOHNSON, S. S., ANSLYN, E. V., GRAHAM, H. V., MAHAFFY, P. R. & ELLINGTON, A. D. 2018. Fingerprinting Non-Terran Biosignatures. *Astrobiology,* 18**,** 915-922.

JOSEPH, A. 2023. Biosignatures—The prime targets in the search for life beyond Earth. *Water Worlds in the Solar System.*

LE VAY, K., SALIBI, E., SONG, E. Y. & MUTSCHLER, H. 2020. Nucleic Acid Catalysis under Potential Prebiotic Conditions. *Chem Asian J,* 15**,** 214-230.

LEOVY, C. 2001. Weather and climate on Mars. *Nature,* 412**,** 245-9.

LI, B. L., ZOU, H. L., LU, L., YANG, Y., LEI, J. L., LUO, H. Q. & LI, N. B. 2015. Size-Dependent Optical Absorption of Layered MoS2and DNA Oligonucleotides Induced Dispersion Behavior for Label-Free Detection of Single-Nucleotide Polymorphism. *Advanced Functional Materials,* 25**,** 3541-3550.

LUNDBLAD, R. L. & MACDONALD, F. M. 2018. *Handbook of biochemistry and molecular biology*, CRC Press.

LUTZ, J. F. 2020. 100th Anniversary of Macromolecular Science Viewpoint: Toward

Artificial Life-Supporting Macromolecules. *ACS Macro Lett,* 9**,** 185-189.

MARTIN, C. & ZHANG, Y. 2007. Mechanisms of epigenetic inheritance. *Curr Opin Cell Biol,* 19**,** 266-72.

MINCHIN, S. & LODGE, J. 2019. Understanding biochemistry: structure and function of nucleic acids. *Essays Biochem,* 63**,** 433-456.

MOJARRO, A., HACHEY, J., BAILEY, R., BROWN, M., DOEBLER, R., RUVKUN, G., ZUBER, M. T. & CARR, C. E. 2019. Nucleic Acid Extraction and Sequencing from Low-Biomass Synthetic Mars Analog Soils for In Situ Life Detection. *Astrobiology,* 19**,** 1139-1152.

MOTOHASHI, K. 2019. Development of highly sensitive and low-cost DNA agarose gel electrophoresis detection systems, and evaluation of non-mutagenic and loading dye-type DNA-staining reagents. *PLoS One,* 14**,** e0222209.

NATH, P., MAHTABA, K. R. & RAY, A. 2023. Fluorescence-Based Portable Assays for Detection of Biological and Chemical Analytes. *Sensors (Basel),* 23.

NEVEU, M., KIM, H. J. & BENNER, S. A. 2013. The "strong" RNA world hypothesis: fifty years old. *Astrobiology,* 13**,** 391-403.

PEARCE, B. K. D., PUDRITZ, R. E., SEMENOV, D. A. & HENNING, T. K. 2017. Origin of the RNA world: The fate of nucleobases in warm little ponds. *Proc Natl Acad Sci U S A,* 114**,** 11327-11332.

PFLUGRADT, R., SÄNGER, T., SCHLAUDERER, N., HAUSCHKE, D., LUTZ-BONENGEL, S. & SCHMIDT, U. 2012. DNA-Quantifizierung in der forensischen Spurenkunde. *Rechtsmedizin,* 22**,** 237-243.

PIZZARELLO, S. 2007. The chemistry that preceded life's origin: a study guide from meteorites. *Chem Biodivers,* 4**,** 680-93.

POWELL, R. 2012. Convergent evolution and the limits of natural selection. *European Journal for Philosophy of Science,* 2**,** 355-373.

RAYMOND-BOUCHARD, I., MAGGIORI, C., BRENNAN, L., ALTSHULER, I., MANCHADO, J. M., PARRO, V. & WHYTE, L. G. 2022. Assessment of Automated Nucleic Acid Extraction Systems in Combination with MinION Sequencing As Potential Tools for the Detection of Microbial Biosignatures.

Astrobiology, 22**,** 87-103.

SLATKO, B. E., GARDNER, A. F. & AUSUBEL, F. M. 2018. Overview of Next-Generation Sequencing Technologies. *Curr Protoc Mol Biol,* 122**,** e59.

SUMMONS, R. E., ALBRECHT, P., MCDONALD, G. & MOLDOWAN, J. M. 2007. Molecular Biosignatures. *Space Science Reviews,* 135**,** 133-159.

THERMOFISHERSCIENTIFIC. 2008. *Quant-iT PicoGreen dsDNA Reagent and Kits* [Online]. Available: https://assets.thermofisher.com/TFS-Assets/LSG/manuals/mp07581.pdf [Accessed May 1st 2020].

TRAVERS, A. & MUSKHELISHVILI, G. 2015. DNA structure and function. *FEBS J,* 282**,** 2279-95.

WANG, Q., ZHANG, B., XU, X., LONG, F. & WANG, J. 2018. CRISPR-typing PCR (ctPCR), a new Cas9-based DNA detection method. *Sci Rep,* 8**,** 14126.

WASIK, S., SZOSTAK, N., KUDLA, M., WACHOWIAK, M., KRAWIEC, K. & BLAZEWICZ, J. 2019. Detecting life signatures with RNA sequence similarity measures. *J Theor Biol,* 463**,** 110-120.

ZOU, R., WANG, S., CHEN, C., CHEN, X., GONG, H. & CAI, C. 2021. An enzyme-free DNA circuit-assisted MoS2 nanosheet enhanced fluorescence assay for label-free DNA detection. *Talanta,* 222**,** 121505.

Chapter 4: Detection of metabolites as an approach to confirm the existence of extra-terrestrial life

Abstract

The search for extra-terrestrial life is a subject of great interest and has been a focus of scientific research for many years. One of the main approaches to confirm the existence of extra-terrestrial life is the detection of metabolic activity. Metabolites are unique chemical compounds produced by living organisms and provide evidence of metabolic processes. In this context, the detection of metabolites can be used as a tool to confirm the existence of extra-terrestrial life. The successful detection of metabolites on other planetary bodies would provide compelling evidence for the existence of extra-terrestrial life and offer new insights into the diversity and evolution of life in the universe. Furthermore, the study of extra-terrestrial metabolites can also help us understand the conditions and processes that are necessary for the origin and evolution of life and provide information for the search of life on other planets and moons in our solar system and beyond. In this chapter, we reviewed the current state of the art in the detection of metabolites and its application to the search for extra-terrestrial life and proposed optimised methods for detecting thiols, lipids, and amino acids for metabolite detection. Combining simple biochemical assays and mass spectrometry, our findings suggest that the detection of metabolites may prove a powerful tool for the confirmation of extra-terrestrial life and highlights the importance of continued research in this field.

4.1 Introduction

4.1.1 The origin and evolution of metabolism

Metabolism is the sum of enzymatic reactions which take place within cells of living life forms to maintain life by utilizing external energy sources from environments (Peretó, 2011). Among internal enzymatic reactions, gene-encoded enzyme catalysts play an important role in life. Since their stability is limited and constant substitution is required, the enzymes themselves are considered as products of metabolism (Cornish-Bowden, 2004).

The beginning of the biological evolution is considered as containing three necessary prebiotic subsystems: self-reproductive vesicles, self-replicative polymers, and self-maintained chemical networks (Peretó, 2011). Thus, it is important to explain the mechanisms of energy and matter fluxes when describing any complete evolutionary scenario. The protometabolic model proposed by Duve (2005) was outlined that in the early Earth, there was a continuous evolution from the prebiotic phase (using non-genetically instructed catalysts such as minerals and short oligomers) to protometabolism (using genetically instructed catalysts such as RNA), and thioesters played an important role in this process by acting as a bridge between sulphur and phosphate chemistries and energy transduction. These chemical reactions provided the foundation upon which life evolved and one theory is that metabolism came first. The hypothesis from Morowitz (1992) suggested that a primitive form of the reductive tricarboxylic-acid (TCA) cycle was the origin of the prebiotic chemistry which also remain in the most of modern metabolism. The TCA cycle is described as a circular chain of reactions which could produce energy for cells to keep alive by generating a series of precursors of amino acids and lipids participated in the building of membranes and molecules. Ingredients at key points of the TCA cycle, such as citrate, have been identified that they could exist on meteorites and the early oceans on Earth. In the research of Keller et al. (2017), ingredients involved in the TCA cycle was exposed to chemicals which might have been existed in early ocean sediments. When peroxydisulphate, which is a source of highly reactive agents called sulphate radicals, was introduced, a sequence of 24 chemical reactions happened and they were similar to the TCA cycle. This research showed that it is possible to proceed the TCA cycle without enzymatic precursor and when there are sulfate radicals in the environment, the TCA cycle can proceed spontaneously (Keller et al., 2017). The other two fundamental metabolic pathways: pentose phosphate pathway and glycolysis, were also found that can proceed by metal ions rather than the enzymes (Keller et al., 2014). In this study, ferrous iron Fe(II) could catalyse

glycolysis and pentose phosphate pathway-like reactions in earth's earliest oceans and ferrous iron Fe(II) is considered to have high concentrations in the early oceans on Earth. Another point worth highlighting is that in this research, ribose 5-phosphate was detected which is considered as the precursor to RNA and it plays an important role in RNA information encoding, chemical reactions catalysis and replication (Keller et al., 2014, Ralser, 2014).

Considering that metabolism as we know it relies on enzymes, the evolution of enzymes could be explained from the hypothesis of a metal-catalysed metabolism. The theory of early forms of enzymes suggests that metal ions could bind to RNA or short peptides and act as the function of catalysis for early enzymes (Ralser, 2014). The *in vitro* selection experiment designed by Fusz et al. (2005) showed that the complex of RNA and Zn had the ability to catalyse the aldolase reaction.

4.1.2 Alternatives theories for the evolution of metabolic pathways

The evolution of metabolic pathways was considered as the vital step in the development of molecules and cells, which leading primitive life to be better adapted the environment and further growth and reproduction (Fondi et al., 2009). During evolution, gene and operon duplications became the crucial step to produce new genetic material with new metabolic abilities (Fani and Fondi, 2009). According to the RNA world hypothesis, the origin of metabolic pathways might have occurred in the RNA or an RNA/protein world as RNA molecules played an important and necessary role in the early stage of cellular processes **(Figure 4.1)** (Ralser, 2014, ExploringOrigins, 2020). There are several hypotheses proposed to explain the establishment of metabolic pathways which are based on gene duplication. The first one was the Retrograde hypothesis proposed by Horowitz (1945) and suggests that enzymes used in biosynthesis have been generated through gene duplication which occurred in the reverse order found in current pathways. For instance, compound "A" is considered as the first material in the metabolic pathway used by the primordial heterotrophs as well as the final product of the biosynthesis process from precursors "D" to "B" by using the corresponding enzymes **(Figure 4.2)**. When "A" was consumed from the external environment, the cells which could be using enzyme "a" to transform "B" to "A" could survival due to the selective pressure. The gene duplication could allow the selection of variants such as the mutant enzyme "b" which was related to "a" to continue the transformation process from compound "C" to "B" which leading the metabolic pathway became more complexed. This process will continue to proceed until it reached the initial precursor (Figure 2). This Retrograde hypothesis connected

the prebiotic chemistry and the evolution of metabolic pathways and could be used to explained some specific routes (Fani and Fondi, 2009).

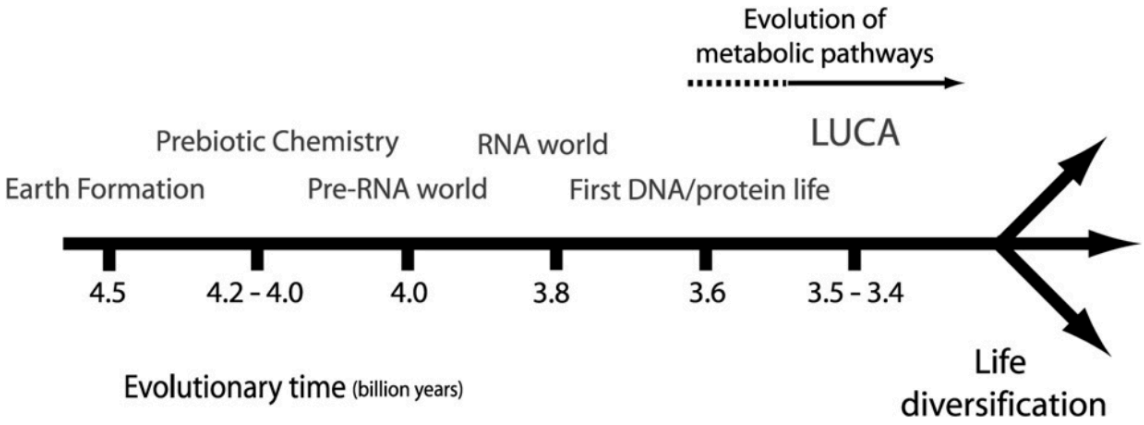


Figure 4.1. Evolutionary time line from the origin of Earth to the diversification of life (Fani and Fondi, 2009).

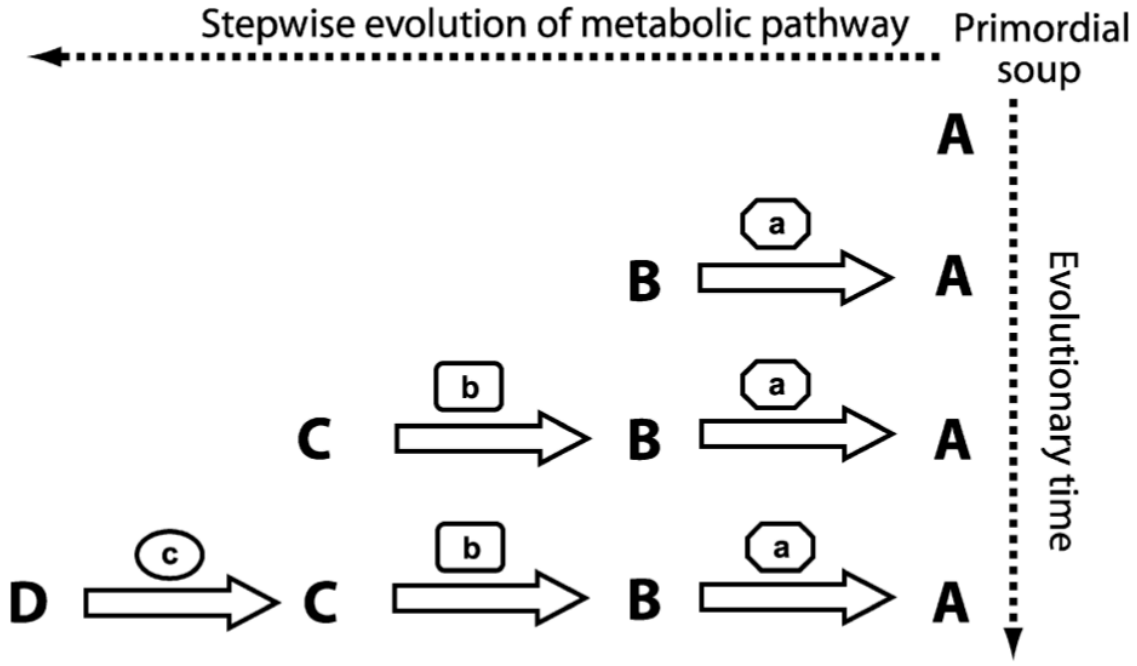


Figure 4.2. Schematic representation of the Retrograde hypothesis on the origin and evolution of metabolic pathways. (Fani and Fondi, 2009).

Contrary to the Retrograde hypothesis, in the research of Granick (1957), an alternative proposal was presented, which suggests that the direction for the evolution of metabolic pathways was forward, where the prebiotic compounds were not the crucial factor to navigate the evolution process (Granick, 1957, Granick, 1965). In this theory, the enzymes for earlier

steps of a metabolic pathway predated the appearance of the latter ones and each intermediate appeared in the metabolic pathway was useful to the cells (Granick, 1957). Finally, a third hypothesis is the patchwork hypothesis proposed by Yčas (1974) and Jensen (1976). This theory explains that metabolic pathways might be constructed by recruiting primitive enzymes which could react with a variety of chemically related substrates. These non-specific enzymes might give primitive cells which have small genomes the capability to overcome the limited coding capabilities. Lazcano and Miller (1996) proposed the theory of the semi-enzymatic origin of metabolic pathways to explain the early metabolic pathways. This theory was based on that some biosynthetic routes could happen spontaneously without enzymes catalysing.

4.1.3 The role of metabolites in the detection of life beyond Earth

Detection of metabolites is crucial for planetary missions as it allows scientists to understand the chemical composition and potential habitability of extra-terrestrial environments. The presence of certain metabolites, such as amino acids and reduced thiols, can indicate the presence of life, while the detection of other metabolites, such as gases and volatile organic compounds, can provide insights into the geochemical processes and atmospheres of planetary bodies. Accurate and sensitive detection of metabolites is essential for verifying the presence of past or present life on other planets, as well as for determining the potential habitability of these environments. By detecting metabolites, scientists can gain a better understanding of the building blocks of life and the conditions required for its formation, which has important implications for astrobiology and the search for extra-terrestrial life. The prospect of detecting extra-terrestrial life has hitherto been approached from various angles. The biological markers known as metabolites represent one such avenue of exploration, providing a unique and critical perspective on this profound astrobiological question.

Metabolites, by definition, are the intermediates and products of metabolism, the network of chemical reactions that maintain and regulate life's function at the cellular level (Judge and Dodd, 2020). Metabolites range from small, simple molecules like water and carbon dioxide to complex organic compounds such as lipids, amino acids, and nucleic acids. These molecules provide direct evidence of life's biochemical activity, making them invaluable in the search for extra-terrestrial life. Unlike macroscopic or even microscopic signs of life, which may be ephemeral or elusive, metabolites pervade an organism's surroundings, becoming ingrained in the environment (DeBerardinis and Thompson, 2012). They offer a means of detection even when life forms are not immediately present or visible. Importantly, these metabolites can

remain stable over extended periods, becoming 'fossilized' within the geological matrix and preserving a record of past life (Westall and Cavalazzi, 2011).

Moreover, the unique chemical signatures and isotopic ratios of biologically produced metabolites often differ from those produced through abiotic processes (Pizzarello, 2006). Consequently, the detection of certain metabolites with characteristic signatures could provide compelling evidence for biological activity beyond Earth. For instance, the presence of a suite of chiral organic molecules - which can be preferentially produced by biological processes - may serve as robust biosignatures (Lee et al., 2022). The detection of metabolites and their study, therefore, has the potential to offer a wealth of information about possible extra-terrestrial life forms. It can provide clues about their metabolic pathways, the complexity of their biochemistry, and the environmental conditions in which they thrive. Such information may, in turn, inform our understanding of life's potential resilience and adaptability in seemingly hostile extra-terrestrial environments (Vemuri and Aristidou, 2005).

4.1.4 Holistic approaches to detect metabolites

Metabolomics, also described as metabolite profiling, entails the comprehensive study of the metabolome, the collective ensemble of small molecular entities within cells, tissues, biofluids, or entire organisms (Theodoridis et al., 2011, EMBL-EBI, 2020) and the aim of metabolomics is to generate holistic metabolic profiles from complex samples, such as tissue extracts or biological fluids (Theodoridis et al., 2012). Achieving this objective necessitates the utilization of a suite of analytical technologies, including but not limited to nuclear magnetic resonance (NMR) spectroscopy, gas chromatography and liquid chromatography both coupled with mass spectrometry (GC-MS and LC-MS, respectively), and capillary electrophoresis. These techniques are instrumental in obtaining the most exhaustive representation of the metabolome (Theodoridis et al., 2011).

NMR spectroscopy is considered as an effective technique for metabolite profiling, and it can be directly used to identify and quantify a variety of analytes. But the complex operation and the high cost of the initial acquisition for the spectrometer lead to the amount of NMR equipment available for metabolomics work is limited (Coen et al., 2008). GC-MS is also an important technique in the holistic metabolite analysis as its high sensitivity for the analysis of volatile and semi-volatile analytes. Compared with other techniques, the GC-MS has its own electron impact (EI) spectral libraries which could provide identification (or annotation) to unknown biomarkers (Kanani et al., 2008). However, the limitation of GC-MS is that the poor

ability of analysing polar, non-volatile analytes, macromolecules and thermolabile compounds which restricts it coverage (Theodoridis et al., 2011). As most of the metabolites in biological samples are involatile, the LC-MS provides better analytical coverage. Major advantages of LC-MS include its higher sensibility compared to NMR and its application to both semi-volatile and non-volatile compounds at the same operation (Theodoridis et al., 2012, Plumb et al., 2002), and therefore, make LC-MS an essential component metabolomic analyses.

Capillary electrophoresis is a new introduced technique for metabolite profiling. It is considered as a high-resolving method but is not fully utilized for complex samples (Maier and Schmitt-Kopplin, 2016). There are several advantages of using capillary electrophoresis in metabolites analysis, such as low solvent consumption and low cost of fused-silica columns using for separation (Tomás-Barberán, 1995). Due to the property of electrophoretic techniques, capillary electrophoresis works well in analysing charged molecules, such as nitrogen containing metabolites. However, the limitation of this technique is the reduced sensitivity compared with main separation techniques (LC-MS, GC-MS). Hence, coupling with mass spectrometry (capillary electrophoresis-mass spectrometry) could be optimised this technique, especially for analysing polar ionogenic compounds (Zhang et al., 2017).

Reduced thiols are organic compounds that contain a sulfur atom and have a free electron available for bonding. They are produced by many living organisms as part of their metabolic pathways and can provide evidence for the presence of living organisms or metabolic processes in extra-terrestrial environments. The presence of reduced thiols in such environments would indicate the potential for life to exist and thrive on other planetary bodies, providing valuable information for astrobiology and the search for extra-terrestrial life (Mora et al., 2013). The common methods used for the detection of thiols include liquid chromatography (LC), gas chromatography (GC) and Capillary Electrophoresis (CE) (Toyo'oka, 2009). All these methods can be equipped with mass spectrometry (MS) for the subsequent analytical testing. Among these methods, GC-MS has been used for in situ planetary exploration, although it is only capable of analysing volatile chemicals, which are often formed when samples are heated to an extreme temperature (pyrolyzed) (Mahaffy, 2007). Another optimised approach is to use microchip CE (μCE), which could be considered as a complementary technique for GC-MS. It enables analysis of a wider variety of thiols without the need to heat the sample (Mora et al., 2013). The μCE technique detects thiols with the commercial fluorescent probe Pacific Blue C5-maleimide, and then separates them using Micellar electrokinetic chromatography (MEKC). By analysing the complex samples from the geothermal pools at Hot Creek Gorge near

Mammoth Lake, California, it showed that 12 thiols were assessed in less than 2 minutes with sensitivities in the low nM range (1-15 nM) after a 2 h labelling step (Mora et al., 2013). However, prior to use this technique, there is a series of essential steps need to be taken: the separation channel needs to be adjusted by NaOH, water and buffer; different level of electricity needs to be applied to buffer, sample, sample waste, and waste. Cumbersome steps are detrimental to the accuracy of the results, especially when run the method in space.

4.1.5 Limitations and challenges in metabolite detection

The prospect of detecting metabolites as indicators of extra-terrestrial life, while tantalizing, is fraught with significant challenges and limitations. The complexity of such endeavours necessitates a meticulous and comprehensive exploration of the associated obstacles.

One of the pivotal challenges lies in the issue of micro-sample handling and contamination (Summons et al., 2008). Earth-based biological and chemical contaminants could inadvertently be introduced during the manufacturing, assembly, launch, or operation of space-bound instruments. These terrestrial contaminants may confound the detection and analysis of potential extra-terrestrial metabolites, resulting in inaccurate or misleading results (Bhattaru, 2018). Extensive sterilization protocols, contamination control measures, and robust data interpretation methodologies are therefore essential to minimize such risks and ensure the reliability of findings (Rummel, 2001). However, even with precautions, absolute prevention of contamination may not be feasible due to the minute nature of metabolites and the high sensitivity of detection instruments (Wolfender et al., 2015).

Metabolite degradation over time presents another significant challenge. The harsh and volatile conditions in outer space environments – such as radiation exposure, extreme temperatures, and chemical interactions – could lead to the decomposition or alteration of metabolites (Bornman et al., 2019). This degradation might render some metabolites undetectable or distort their true nature, potentially leading to misinterpretation of data (Fell and Cornish-Bowden, 1997). For example, due to the complex and extreme environment on Mars, soil samples on the surface of Mars could have been exposed to unpredictable chemical reactions and their molecular biosignatures could be corrupted or lost entirely. The research of Navarro-Gonzalez et al. (2006) showed that the soil compounds on Mars could be oxidized by heating through the GC-MS analysing which causes the problem of the sample's preservation and detection. Consequently, it is crucial to account for environmental conditions and potential degradation pathways in metabolite analysis. Development of enhanced detection methods that can identify

degraded or altered metabolites will also play a pivotal role in overcoming this challenge.

Furthermore, the inherently intricate nature of metabolites and their diverse biological pathways adds another layer of complexity. Metabolites can be the products of numerous biological processes, and their presence does not necessarily indicate life – they could also arise from abiotic processes (Bapat and Rajamani, 2023). Additionally, some metabolites may not be associated with life as we know it, but rather, with hypothetical, unfamiliar forms of life (Pross, 2016). These complexities necessitate a comprehensive and nuanced understanding of both terrestrial and potential extra-terrestrial biological processes.

In conclusion, while the detection of metabolites holds great potential for confirming the existence of extra-terrestrial life, it is also a venture replete with formidable challenges. Addressing these limitations requires a combination of methodology, technology, and a nuanced understanding of both life on Earth and potential life beyond our planet.

4.1.6 Structure of the chapter

The emphasis of this chapter is on the development and optimization of simple, biochemical methods for metabolite detection, specifically targeting thiols, lipids, and amino acids.

The initial section focuses on thiols, specifically glutathione and cysteine, due to their crucial role in oxidative stress and maintaining normal cellular metabolism. The section details the process of developing optimal spiking conditions and determining recovery rates, highlighting the effectiveness of methanol as a spiking solvent. This section also explores the impact of UV radiation and low temperature on thiol stability, offering insights into potential thiol existence beneath the Martian surface.

The subsequent section examines lipids, complex compounds that can serve as life signals. This part of the chapter outlines the process of troubleshooting lipid detection methods, focusing on the effective use of chloroform for spiking and quantification via the vanillin assay. In addition, this section delves into the effects of UV radiation and low temperature on lipids and the protective role of soil simulants and clays against UV degradation.

The final section investigates the role of amino acids as essential components of life. It outlines a simplified extraction method using water on amino acid-spiked soil simulants, followed by analysis through HPLC and mass spectrometry. This section discusses the capability of this method to detect a comprehensive spectrum of amino acids from soil simulants.

4.2 Materials and Methods

4.2.1 Metabolites for the experimental design and subsequent analyses

The primary metabolites selected for this study encompassed a broad range of chemical classes, each serving distinct yet interconnected functions within cellular metabolic processes.

Reduced thiols were represented by glutathione (GSH), selected due to its critical role in cellular defence against oxidative stress and maintenance of redox homeostasis (Aoyama and Nakaki, 2015) and its presence could potentially indicate the existence of life with similar oxidative protection mechanisms. This selection was further underpinned by its characteristic thiol (-SH) group, which allowed GSH exhibited unique chemical properties that allow it to engage in specific interactions, making it identifiable by advanced detection techniques (Forman et al., 2009).

Further, the study incorporated the use of cysteine, another reduced thiol of significant biological relevance. Given its status as a semi-essential amino acid and its involvement in protein synthesis and detoxification processes, cysteine offered additional depth to the metabolic profile being investigated (Combs and DeNicola, 2019).

The lipid component of this study was represented by krill lipid. Krill lipids are notable for their high concentrations of phospholipids and omega-3 fatty acids, constituents crucial to cellular membrane function and various metabolic processes (Burri et al., 2012). Moreover, krill lipid can offer insights into the conditions and metabolic pathways that might support life in extreme environments. Krill are able to survive in the harsh conditions of the Antarctic ocean, suggesting their lipids may have unique structural features or adaptations that could offer valuable information for astrobiological studies (Hellessey et al., 2020). As lipids are fundamental to cellular life, the incorporation of krill lipids provided a means to investigate the preservation and detectability of such compounds under potential extra-terrestrial conditions.

Amino acids, the building blocks of proteins, were also included in the study. Their ubiquitous presence in terrestrial life, along with their role in various biological processes, reinforced their relevance to the research (Lopez and Mohiuddin, 2020).

Finally, Fatty Acid Methyl Esters (FAMEs) were utilized in this research. As derivatives of fatty acids, FAMEs served as reliable proxies for the parent lipids from which they are derived. FAMEs were prepared by transesterification of lipids, a process that makes the long-chain fatty

acids more volatile and amenable to analysis by gas chromatography-mass spectrometry (GC-MS). This allowed for a detailed characterization of the original lipid profile in terms of chain length and saturation level, aiding in the identification of complex lipid mixtures (Xu et al., 2010). Moreover, FAMEs were more stable than their parent fatty acids, resisting oxidative degradation (Pullen and Saeed, 2012). This is particularly important when considering the harsh conditions of extra-terrestrial environments where biomarkers must remain stable over extended periods for detection. The use of FAMEs provided insights into the fatty acid composition of the examined samples and added another dimension to the metabolic profile. Given their relative stability and ease of detection, FAMEs served as valuable markers in this study. Collectively, these materials formed the basis for a comprehensive and nuanced approach to the investigation of potential biomarkers for extra-terrestrial life.

4.2.2 Spiking thiols on the Martian soil simulant and their extraction and quantification

Reduced glutathione (GSH) was spiked on the Martian soil simulant as describe in previous chapters. Reduced thiols were extracted in ddH_2O and quantified using the aldrithiol reaction. This method was based on the research of Grintzalis et al. (2014) which stated that the photometric reagent 4,4'-dithiopyridine (DTP) can react with thiols through a thiol-disulfide exchange reaction to produce the 4-thiopyridone which can be detected under the absorbance at 325 nm (**Figure 4.3**). In this chapter, 4,4'-dithiopyridine (DTP) was used as the thiol probe to detect reduced thiols by reacting with GSH. DTP was prepared in DMSO (30 mM) and diluted to 0.75 mM DTP in 100 mM acetic acid pH 4.5. A series of GSH standards (2-20 µM) were prepared in 100 mM acetic acid pH 4.5. The samples and standards were assayed for thiols concentration according to **Table 4.1**.

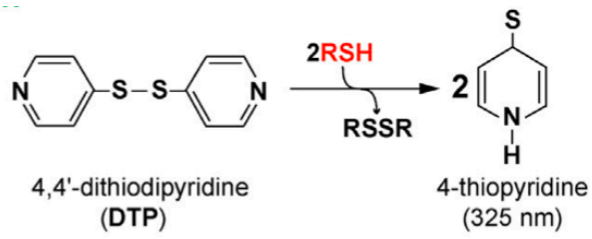


Figure 4.3. Mechanism of thiol quantification by the aldrithiol reaction (Grintzalis et al., 2014).

Table 4.1. Reduced thiols assay conditions. The volumes are in µl. RB: Reagent Blank, S: Sample or Standard, SRB: Sample or Standard Reagent Blank, SB: Sample or Standard Blank.

Reagents	**RB**	**S**	**SRB**	**SB**
Sample appropriately diluted in 100 mM acetic acid pH 4.5	-	200	-	200
100 mM acetic acid pH 4.5	200	-	200	-
0.75 mM DTP	50	50	-	-
100 mM acetic acid pH 4.5	-	-	50	50

The mixtures were incubated for 10 minutes at room temperature and the absorbance was measured at 325 nm. The net absorbance derived from the absorbance difference of S-RB-(SB-SRB) was converted to GSH concentration equivalents using the corresponding standard curve.

4.2.3 Spiking cysteine on the Martian soil simulant and its extraction and quantification

Cysteine is one of the amino acids which contains the thiol side chain. Due to the existence of a thiol group, it can be oxidated to the disulphide derivative cystine. Based on the research of Grintzalis et al. (2014), ninhydrin (NHD) can be used to quantify cysteine as it can react with the thiol part in cysteine (**Figure 4.4**). In this chapter, ninhydrin was used to detect and quantify cysteine. Ninhydrin (60 mM) was prepared in the mixture of acetic acid and HCl (acetic acid: HCl, 1.5: 1). An original cysteine stock (10 mM) was prepared in 0.1 N HCl and then a series of cysteine standards (5-50 µM) from the original stock were prepared in ddH_2O. The samples and standards were assayed for cysteine concentration according to **Table 4.2**.

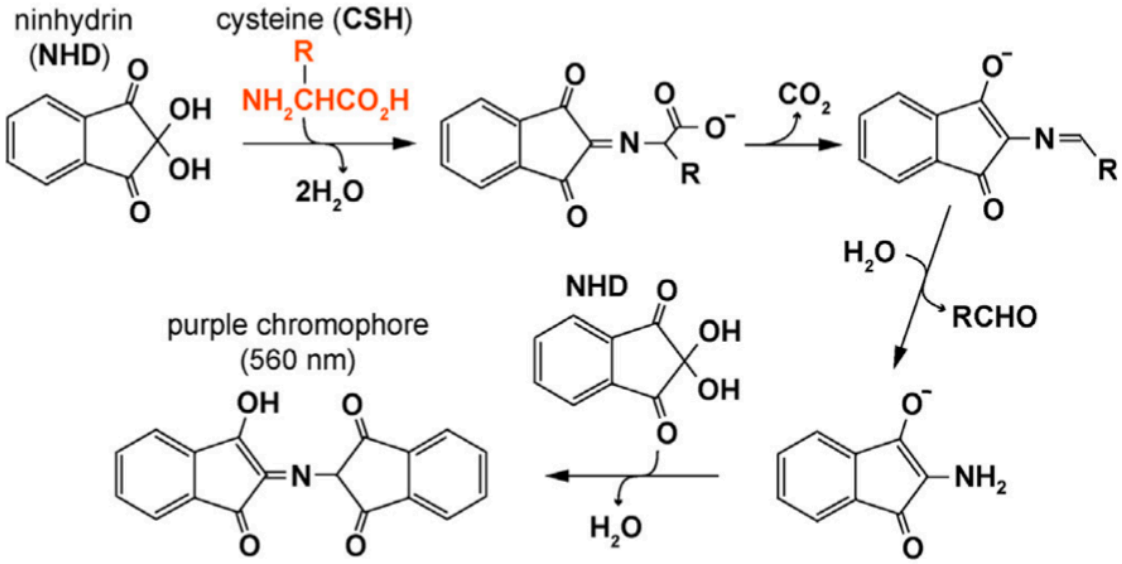


Figure 4.4. Mechanism of cysteine quantification by the ninhydrin reaction (Grintzalis et al., 2014).

Table 4.2. Quantification of cysteine assay conditions. The volumes are in µl. RB: Reagent Blank, S: Sample or Standard.		
Reagents	**RB**	**S**
Sample appropriately diluted in ddH_2O	-	200
ddH_2O	200	-
60 mM ninhydrin	200	200

The mixtures were incubated for 10 minutes at 100 °C in water bath, then 200 µl ice-cold methanol was added into the mixtures to stop the reaction. The absorbance was measured at 560 nm. The net absorbance derived from the absorbance difference of S-RB was converted to cysteine concentration equivalents using the corresponding standard curve.

4.2.4 Spiking of lipids on Martian soil simulant and their extraction and quantification

Lipids are also considered as the potential metabolites which may exist on Mars. Krill is a complex mixture of lipids which was spiked on the Martian soil simulant similar to other chapters. Following, lipids were extracted in chloroform and dried. The dried extracts were re-suspended in 18 M H_2SO_4 and incubated at 96 ºC for 15 minutes. Then the reagents of vanillin and mixture of sulfuric and phosphoric acid were added according to **Table 4.3**. For the reagents, vanillin was prepared at 10 mg/ml methanol. For the vanillin sulfuric phosphoric mixture, vanillin, 85% H3PO4, 18 M H2SO4, and water were mixed with a ratio of vanillin 10

mg/ml methanol: H3PO4: 18 M H2SO4:ddH2O (1:1:1:1). As a sample blank reagent a similar mixture without vanillin was prepared.

Table 4.3. Quantification of lipids assay conditions.
The volumes are in µl. RB: Reagent Blank, S: Sample or Standard.

Solutions	Sample	Sample Blank	Reagent Blank	Sample Reagent Blank
Sample dissolved in 18 M H_2SO_4	50	50	-	-
18 M H_2SO_4	-	-	50	50
Vanillin 10 mg/ml methanol: H_3PO_4: 18 M H_2SO_4:ddH_2O (1:1:1:1)	200	-	200	-
Methanol: H_3PO_4: 18 M H_2SO_4:ddH_2O: (1:1:1:1)	-	200	-	200

The mixtures were incubated for 15 minutes at room temperature and the absorbance was measured at 595 nm. The net absorbance derived from the absorbance difference of S-RB-(SB-SRB) was converted to lipid (Krill) concentration equivalents using the corresponding standard curve.

4.2.5 Spiking and extraction of amino acid on Martian soil simulant and detection with chromatography

4.2.5.1 Detection with chromatography coupled with mass spectrometry without derivatisation

A mixture of 20 mM amino acids was spiked with 50 mg soil matrix and dried. Following the soil matrix was extracted in 200 µl LC-MS water or 80% LC-MS methanol: LC-MS 20% water and the extracts were centrifuged to clear from soil debris and vacuum dried using a speedvac until analysis. Samples were re-suspended in 300 µl of 95% LC-MS water: 5% LC-MS methanol, vortexed, centrifuged and the clear supernatant was collected. 10 µl were analysed by HPLC (Thermo Ultimate 300 system) coupled to a QExactive Plus mass spectrometer (Thermo). For the chromatography, a PFP column (Thermo Accucore, PFP, 150x 2.0 mm, 2.6 µm) was used with mobile phase A of 0.1% formic acid and mobile phase B of methanol containing 0.1% formic acid. After injection of 10 µl, eluent A was held for 5 min at 100%, flowed by a linear increase to 95% B for 5 min, an isocratic elution at 95% B for 2 min, and a re-equilibration to the initial conditions for 5 min. Analytes were detected after electrospray ionization in positive ion mode as M^+H^+ ions.

4.2.5.2 Detection with chromatography with derivatisation

The present study employed an analytical methodology that involved spiking 50 mg soil matrix with a mixture of 2 mM amino acids, subjecting half of this sample to a UV radiation exposure of 72 kJ/hm2 for a duration of 6 hours. Subsequent to this treatment, the extraction of free amino acids was conducted on both the control (unirradiated) and UV-irradiated soil samples. This extraction process involved a 10-minute ultrasonic bath treatment with 0.4 ml of 0.1 M HCl at 4°C, followed by centrifugation at 16,400 g for 10 minutes to effectively remove cellular debris. Derivatization of the amino acids was achieved utilizing AccQ-Tag reagent (Waters). The derivatized products were subsequently subjected to reverse-phase chromatographic separation on an Acquity BEH C18 column (150 mm × 2.1 mm, 1.7 µm, Waters), linked to an Acquity H-class UPLC system. Quantification of the amino acids was achieved via fluorescence detection utilizing an Acquity FLR detector (Waters). The analytical column was maintained at a temperature of 42°C and pre-equilibrated with five column volumes of Buffer A (consisting of 140 mM sodium acetate at pH 6.3, and 7 mM triethanolamine) at a flow rate of 0.45 ml/min. The successful baseline separation of amino acid derivatives was facilitated by progressively increasing the concentration of acetonitrile (Buffer B) in Buffer A, as follows: 1 min at 8% B, 7 min at 9% B, 7.3 min at 15% B, 12.2 min at 18% B, 13.1 min at 41% B, 15.1 min at 80% B, a hold phase of 2.2 min, and a return to 8% B within 1.7 min. The Empower3 software suite (Waters) was utilized for data acquisition and processing (Yang et al., 2015).

4.2.6 Spiking and extraction of lipids on Martian soil simulant and detection with chromatography coupled with mass spectrometry

For the detection of lipids with mass spectrometry, 50 µl 10 mg/ml FAMEs were spiked with 50 mg soil matrix and dried. Then under UV radiation for 6 hours. Then Soil samples were extracted by adding 400 µL 100% methanol (HPLC-grade), vortexing and incubating for 10 min. on ice. After the addition of 100 µL 100% chloroform (HPLC-grade), samples were vortexed and incubated for 5 min on ice. To remove soil particles, extracts were filtered through glass fiber filter units by centrifugation at 2,000 xg. To separate polar and organic phases, 200 µL HPLC-grade water were added and samples were centrifuged for 10 min at 11,000x g.

To analyze total fatty acids, 80µL of the lower organic phase after extraction were transferred to a glass vial and dried in a speed-vac without heating. For transmethylation reactions, pellets were re-dissolved in 40µL TBME (tert-Butyl methyl ether, Sigma) and 20µL TMSH (Trimethylsulfoniumhydroxid, Sigma), incubated for 45 min. at 50°C and analyzed using a

GC/MS-QP2010 Plus (Shimadzu®) fitted with a Zebron ZB 5MS column (Phenomenex®; 30 meter x 0.25 mm x 0.25 µm) for fatty acid methyl esters (FAME). The GC was operated with an injection temperature of 230°C and 1µL sample was injected with split mode (1:20). The GC temperature program started with a 1 min. hold at 40°C followed by a 6°C/min ramp to 210°C, a 20°C/min ramp to 330°C and a bake-out for 5 min. at 330°C using Helium as carrier gas with constant linear velocity. The MS was operated with ion source and interface temperatures of 250°C, a solvent cut time of 7 min and a scan range (m/z) of 40–700 with an event time of 0.2 sec. The "GCMS solution" software (Shimadzu®) was used for data processing.

4.3 Results

4.3.1 Development of a method for the quantification of thiols on soil matrices

In order to optimize the method of reduced thiols detection by using DTP, several approaches were explored in relation to the solvent used for spiking, the method of evaporation (on soil) and the solvent for extraction. Initially, two options of spiking solvent (methanol and 100 mM acetic acid pH 4.5) were explored for spiking thiols standards of GSH. GSH standards (500 µM), which were prepared in methanol and acetic acid, were spiked equally on per 50 mg soil matrix and evaporated using an oven at 37 °C for 24 hours (**Figure. 4.5**). Then the spiked soils were collected and then extracted in ddH_2O or acetic acid. The extraction was reacted with DTP and measured the absorbance at 325 nm. The first observation was that there was an extremely statistically significant difference between using methanol and acetic acid as the solvent used for spiking, which indicated that methanol can be a better option as a solvent to spike GSH than acetic acid, as it can get a higher absorption of reduced thiols by spiking in methanol.

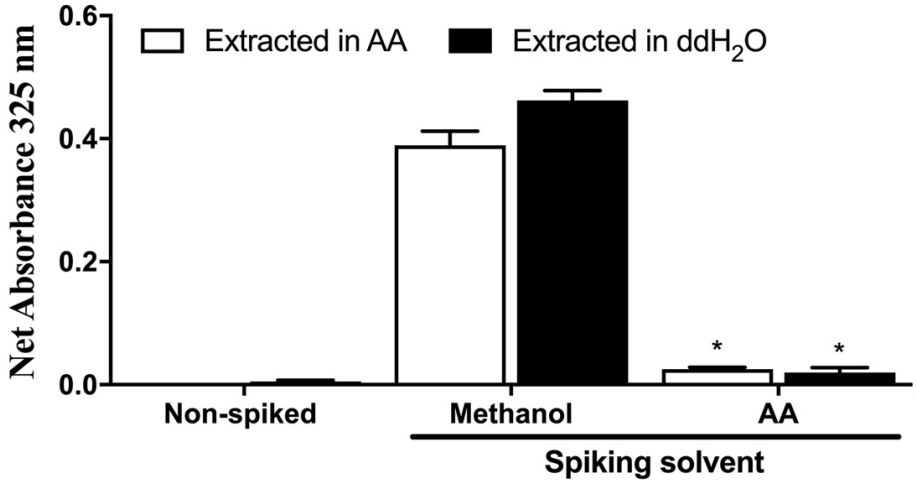


Figure 4.5. Exploring the difference in spiking solvent for spiking GSH. Data represent average±SD. AA: 100 mM acetic acid pH 4.5. *Statistically significant by Student's *t*-test.

Following, the method of evaporation (on soil) was explored for alternatives using oven compared with speed vacuum concentrator. 500 µM GSH dissolved in methanol was spiked on soil in Eppendorf tubes (dried in either the oven at 37ºC or speed vacuum concentrator) and then extracted in ddH_2O or acetic acid. The reduced thiols were assessed by the DTP method (**Figure 4.6**). The result showed that using speed vacuum concentrator can recover more GSH from the soil. The other conclusion from this result was using ddH_2O to extract the spiked soil

can get a higher absorbance.

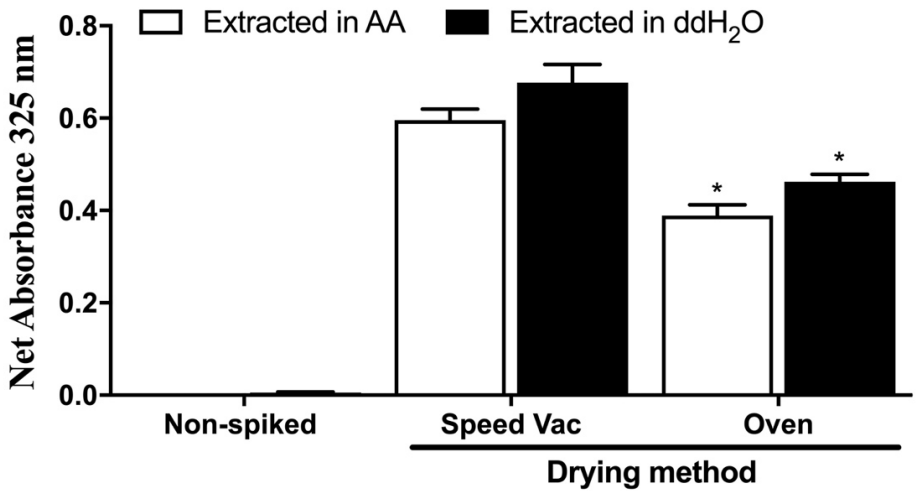


Figure 4.6. Drying soil in the speed vacuum concentrator or the oven at 37 ºC using Eppendorf tubes. Data represent average±SD (N=3). AA: 100 mM acetic acid pH 4.5. *Statistically significant by Student's *t*-test.

Having optimized all the assay parameters mentioned above, a series of different concentrations (from 2 to 20 μM) of GSH was measured as the standard curve of the assay (**Figure 4.7**).

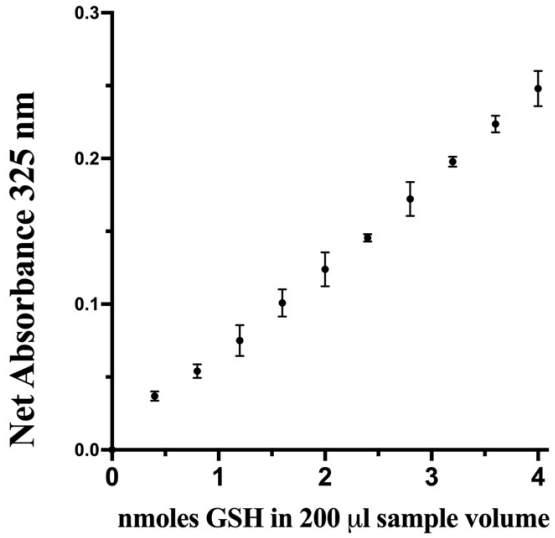


Figure 4.7. Linear standard curve of GSH from 0.4 to 4 nmoles in 200 μl sample volume. Data represent average±SD (N=3).

Then the recovery of the GSH from the spiked soil was explored. GSH-spiked soil simulants

were extracted in water and then compared with the same concentration of pure GSH solution (**Figure 4.8**). 50 nmoles GSH (50µl from a 1 mM GSH stock in methanol) was spiked in 50 mg soil simulants and extracted in 1 ml water (in total). The result showed the recovery of spiked-GSH from soil simulants was from 72.5% to 92.7% among in four times extractions. With the increase of the number of extractions, the recovery of GSH is getting higher.

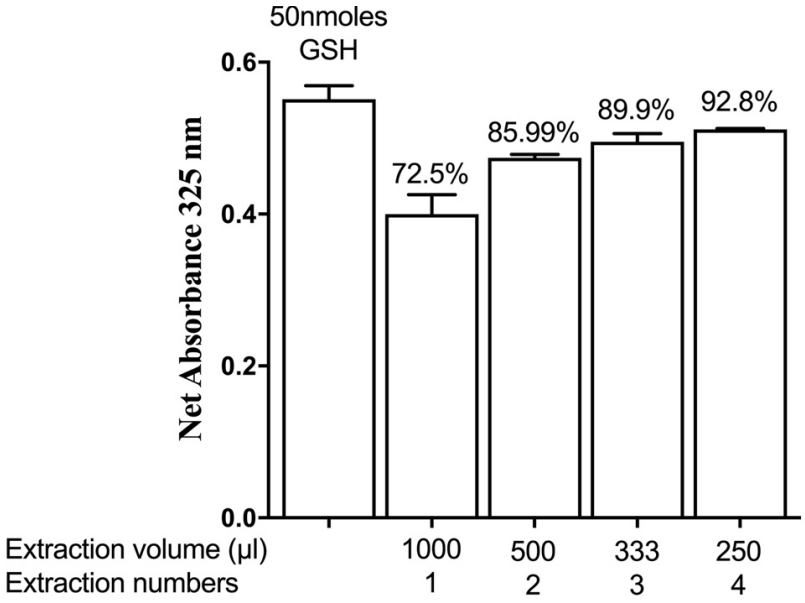


Figure 4.8. The recovery of GSH from spiked soil simulants. Data represent average±SD (N=3).

Following, a series of concentration of GSH-spiked soil matrix and the reduced thiols standards were tested in spiked soil matrix by DTP assay (**Figure 4.9**). Since in a more realistic case scenario which UV radiation on the surface of Mars would result in adverse conditions for the existence of metabolites, the spiked soil matrix was also exposed to UV radiation at the UV dosage of 72 kJ/hm^2 for 4 hours (**Figure 4.10**). UV radiation resulted in a significant decrease of the signal detected, after four hours exposure to UV, the signal can be decreased around 35±2%, which could be attributed to the decomposition of thiols spiked on the soil matrix.

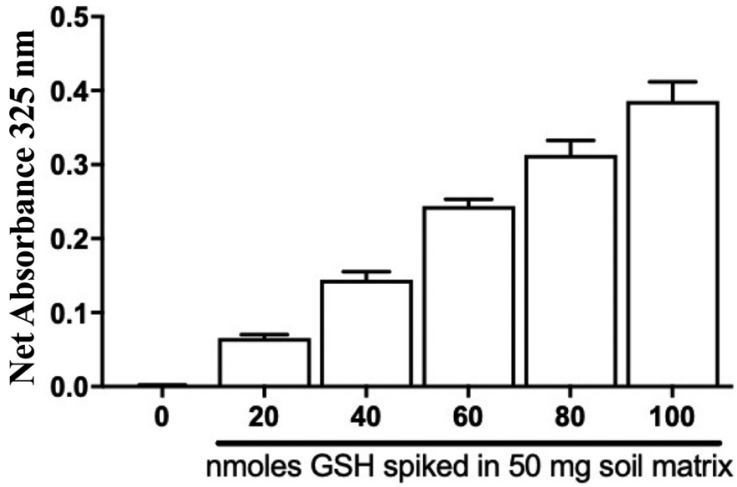


Figure 4.9. Quantification of reduced thiols (standards of GSH) spiked on the soil matrix. Data represent average±SD (N=3).

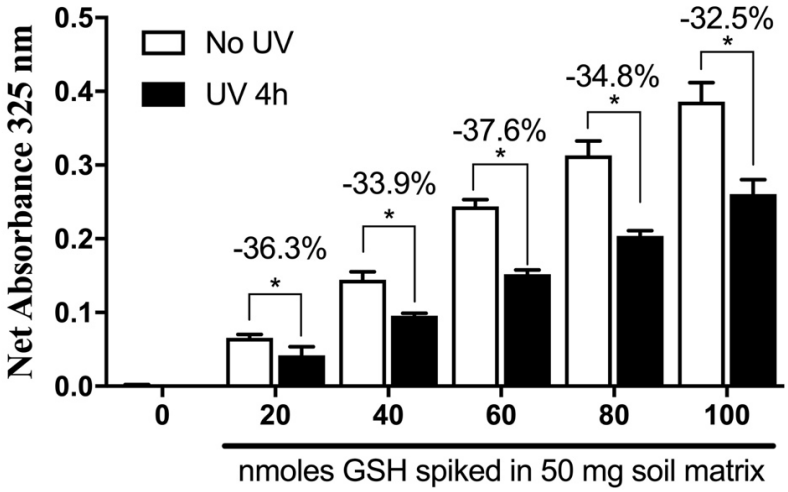


Figure 4.10. The impact of UV radiation on GSH-spiked soil matrix. Data represent average±SD (N=3). *Statistically significant by Student's *t*-test.

Different clays (Kaolinite (KGa-1b), Na-rich Montmorillonite (SWy-3), Montmorillonite (STx-1b) and Illite-smectite mixed layer (ISCz-1)) were also introduced in this experiment to explore the impact of UV radiation and the recovery (**Figure 4.11**). 50 nmoles GSH (50µl from a 1 mM GSH stock in methanol) was exposed to UV radiation at the UV dosage of 72 kJ/hm^2 for 6 hours. The result indicated that soil/clays could provide to some extent protection from UV radiation, especially KGa-1b, which showed the 27.2% decrease of absorbance signal after 6h UV exposure. There is no significant difference of the protection between JSC soil simulants and other clay samples. Among the soil/clay samples, the ISCz-1 provided a higher recovery (91.69%) and the recoveries for other samples were varied from 13.17% to 85.6%. The reason

for causing the difference in recovery may be due to the physical adsorption and surface chemical activity of the clays.

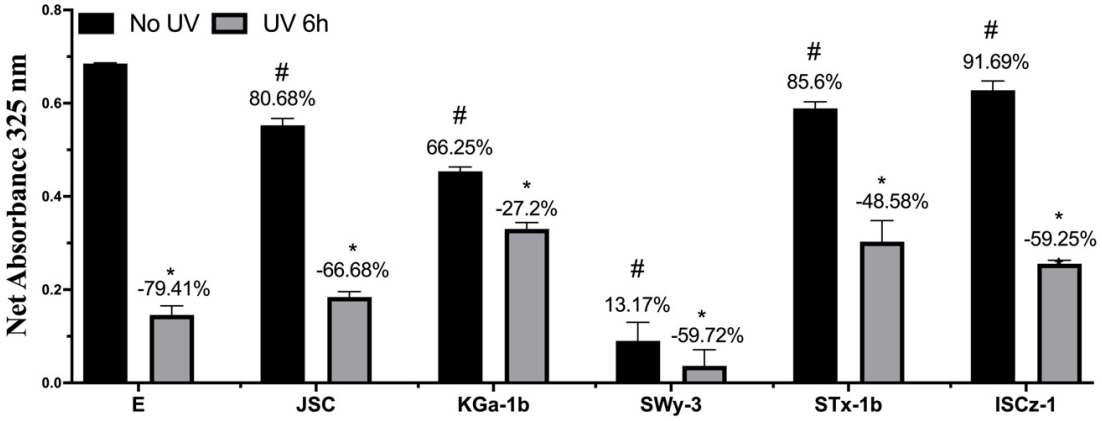


Figure 4.11. The recovery and impact of UV radiation on GSH-spiked soil matrix/clays. Data represent average±SD (N=3). E: no spiking soil/clays. Statistically significant by two-way ANOVA corrected with post-hoc Turkey test. *Impact of UV radiation on samples, # recovery of the GSH signal from soil/clays.

In this chapter, we also adopted a similar approach to the preceding chapter by examining the stability of GSH both in isolation and when spiked into soil samples, under the severe cold temperatures that characterize the Martian environment, particularly at negative 18 degrees Celsius and negative 80 degrees Celsius (**Figure 4.12**). By emulating the extreme temperature conditions varying from the equatorial region to the poles of Mars, this segment provides a comprehensive evaluation of the potential effects of Martian cold on GSH stability. This replication of the Martian environment, in combination with the UV radiation exposure experiments, provides us with a more realistic understanding of the potential degradation or survival of GSH on Mars. The results indicated that the GSH-spiked soil samples exhibited stability under -80-degree conditions, especially in KGa-1b. The resilience of GSH under such extreme cold contributes substantially to our understanding of the potential for the preservation of biological signatures in the Martian environment.

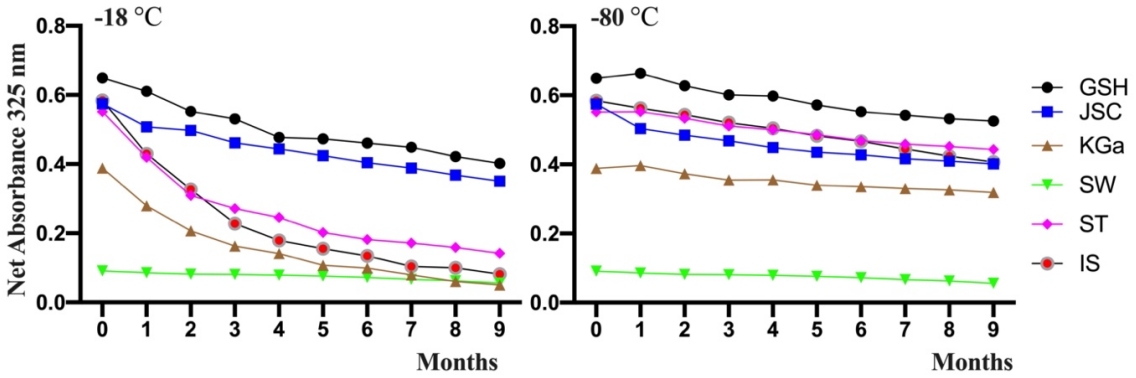


Figure 4.12. Thermal stability of GSH in extreme cold conditions. The figure presents the resilience of both isolated GSH and GSH spiked-soil samples under temperatures of -18°C and -80°C, representative of Mars' harsh cold climate. Data represent average±SD (N=3).

4.3.2 Development of a method for the assay for the quantification of cysteine on soil matrices

Initially, the best incubation time for this assay was explored (**Figure 4.13**). A series of cysteine standards (5-50 μM) were prepared in ddH_2O and were incubated with ninhydrin at 100 °C in water bath for 5, 10, 20 and 30 minutes. Then the ice-cold methanol was added to stop the reaction and the absorbance was measured at 560 nm. The result indicated that with the increase of incubation time, the absorbance will increase, but after 10 minutes, it will drop with lastingness of incubation.

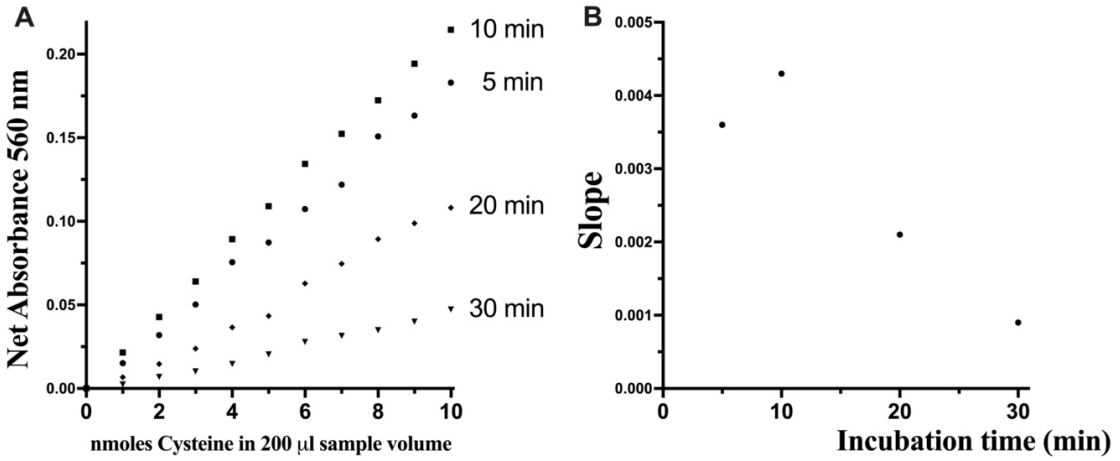


Figure 4.13. The exploration of the best incubation time for quantify cysteine (A. the standard curves of cysteine at different incubation time; B. the slopes from the standard curves at different incubation time). Data represent average±SD (N=3).

Having explored the best incubation condition, a series of different concentrations (from 5 to 50 μM) of cysteine was measured as the standard curve of the assay (**Figure 4.14**). In order to broaden the scope of applications, other amino acids (such as Arginine, Leucine etc.) were also measured at this condition. However, they all did not work by using this method as they do not have thiol chain except Proline. Proline can be measured at a high concentration condition (5-50 mM) (**Figure 4.15**).

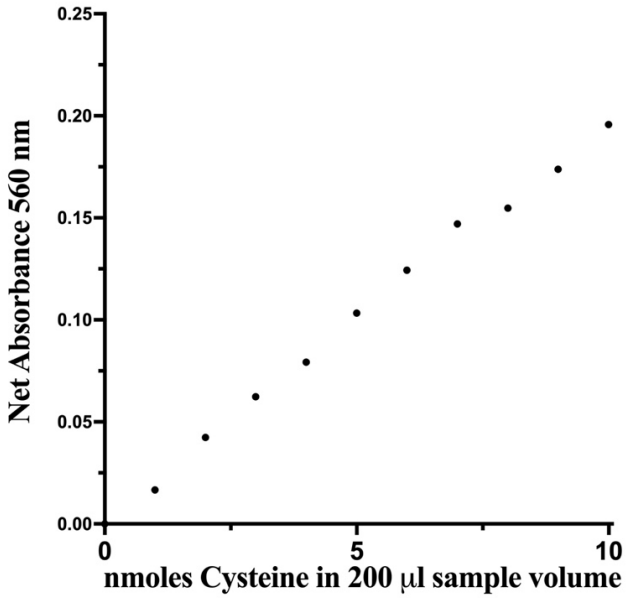


Figure 4.14. Linear standard curve of cysteine from 1 to 10 nmoles in 200 μl sample volume. Data represent average±SD (N=3).

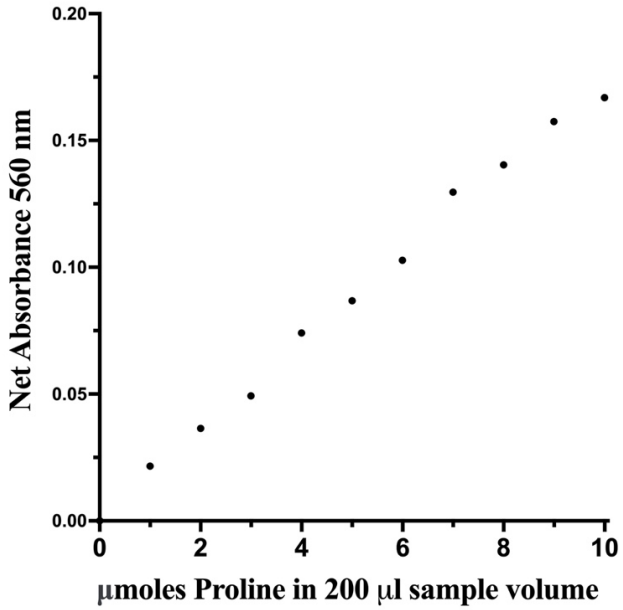


Figure 4.15. Linear standard curve of proline from 1 to 10 µmoles in 200 µl sample volume. Data represent average±SD (N=3).

Different extraction solvent was explored for the recovery of cysteine-spiked soil simulants. 50 mg cysteine-spiked soil was extracted in 1 mM, 10 mM, 100 mM HCl and 1 mM, 10 mM, 100 mM NaOH and then reacted with ninhydrin (**Figure 4.16**). The result indicated that the best option was to use 100 mM HCl to extract cysteine among these solvents.

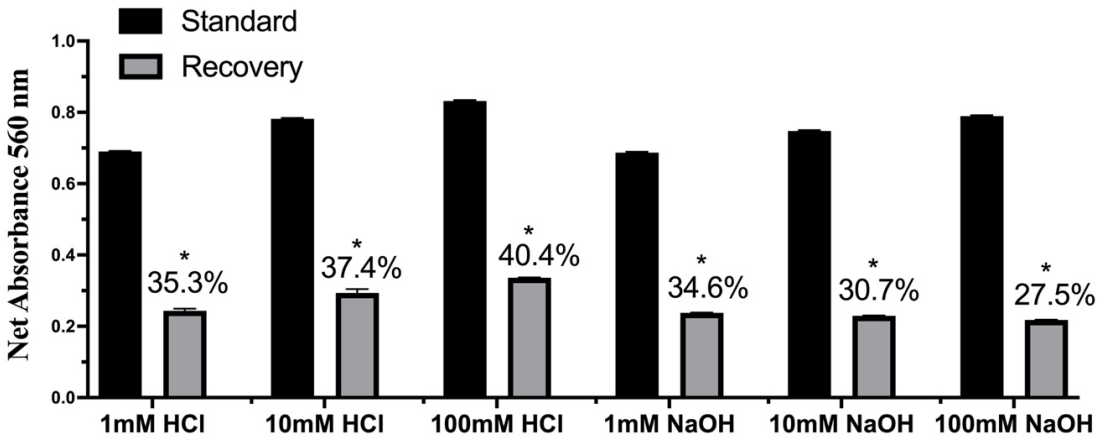


Figure 4.16. The impact of different solvents for extraction of Cysteine-spiked soil matrix. Cysteine-spiked soil was extracted in different solvents and recovered to compared with the standard cysteine. Data represent average±SD (N=3). *Statistically significant by two-way ANOVA corrected with post-hoc Turkey test.

Following, the recovery of the cysteine from the spiked soil was explored. cysteine-spiked soil

simulants were extracted in 100 mM HCl and then compared with the same concentration of pure cysteine solution (**Figure 4.17**). Cysteine from a range of 0.6 mM to 2 mM was spiked in 50 mg soil simulants and extracted in 0.3 ml 0.1M HCl. The result showed the recovery of spiked cysteine can be varied from 28.3% to 40.3% with the increase of the concentration of cysteine.

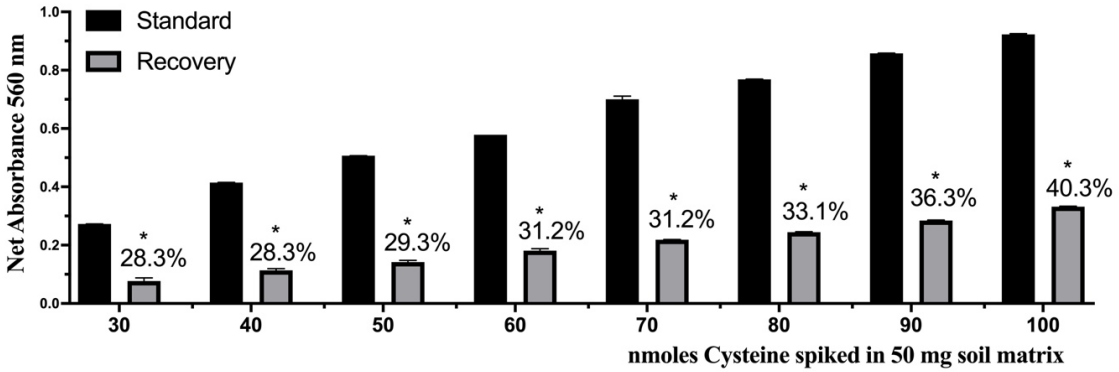


Figure 4.17. The recovery of cysteine from spiked soil simulants. Data represent average±SD (N=3). *Statistically significant by Student's *t*-test.

Following, cysteine was spiked in the soil simulants and tested by using ninhydrin (**Figure 4.18**). The impact of UV radiation was also explored in this cysteine-spiked soil matrix as UV radiation on the surface of Mars would result in adverse conditions for the existence of metabolites. The spiked soil matrix was exposed to UV radiation for up to 120 minutes (**Figure 4.19**). This result indicated that UV radiation caused a significant decrease of the signal detected (after two hours, the signal decreased 56.5%), which could be attributed to the decomposition of cysteine spiked on the soil matrix.

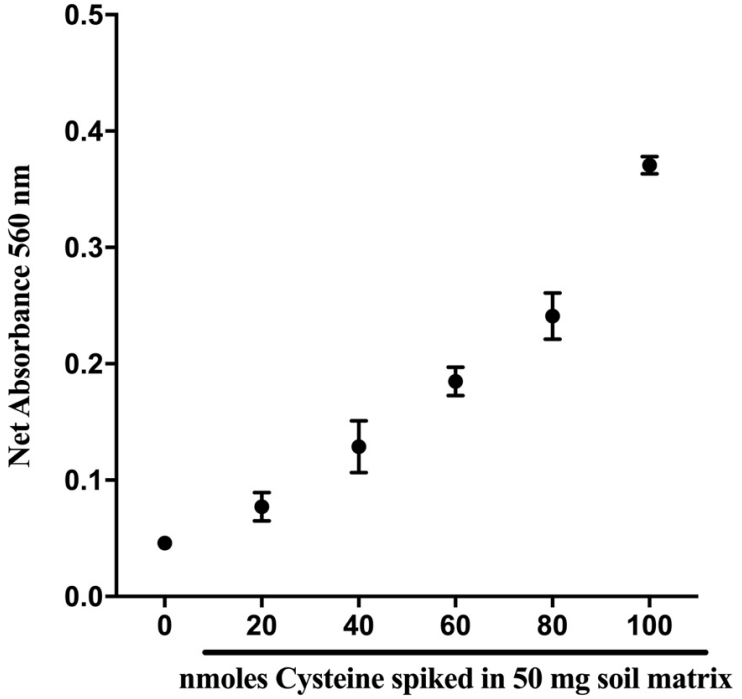


Figure 4.18. Quantification of cysteine (standards of cysteine) spiked on the soil matrix. Data represent average±SD (N=3).

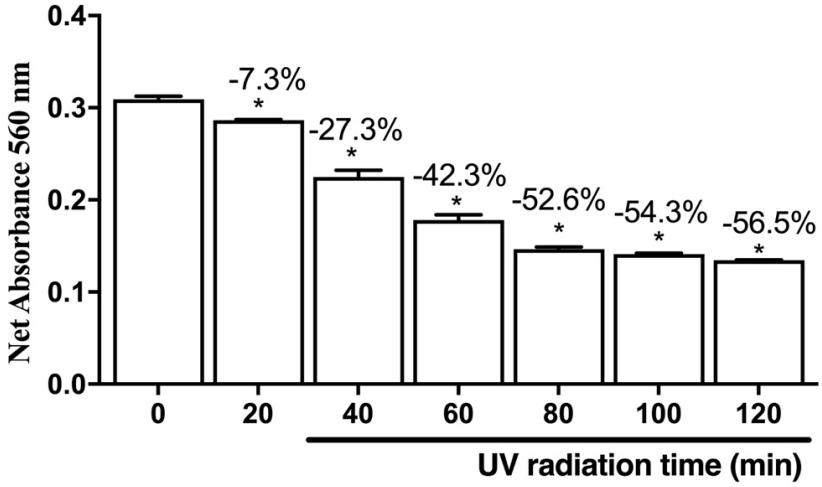


Figure 4.19. The impact of UV radiation on Cysteine-spiked soil matrix. Data represent average±SD (N=3). *Statistically significant by Student's *t*-test.

Similar to GSH, different clays (Kaolinite (KGa-1b), Na-rich Montmorillonite (SWy-3), Montmorillonite (STx-1b) and Illite-smectite mixed layer (ISCz-1)) were also introduced in this experiment to explore the impact of UV radiation and the recovery (**Figure 4.20**). 1 mM cysteine was spiked into soil samples and then exposed to UV radiation at the UV dosage of

72 kJ/hm^2 for 6 hours. The result indicated that soil/clays could provide to some extent protection from UV radiation, especially SWy-3, which showed the 50.2% decrease of absorbance signal after 6h UV exposure while STx-1b cannot protect the cysteine at all (with 98.4% of the decrease of absorbance signal). There is no significant difference of the protection between JSC soil simulants and other clay samples. Among the soil/clay samples, the KGa-1b provided a higher recovery (90.2%) and the recoveries for other samples were varied from 34.1% to 72.5%. The reason for causing the difference in recovery may be due to the physical adsorption and surface chemical activity of the clays.

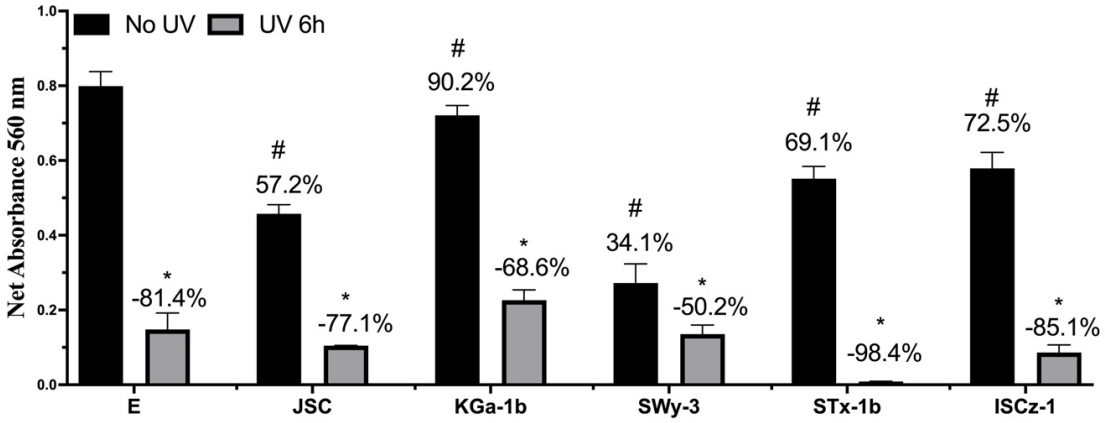


Figure 4.20. The recovery and impact of UV radiation on cysteine-spiked soil matrix/clays. Data represent average±SD (N=3). E: no spiking soil/clays. Statistically significant by two-way ANOVA corrected with post-hoc Turkey test. *Impact of UV radiation on samples, # recovery of the GSH signal from soil/clays.

Through a meticulous investigation into the stability of cysteine, both in isolation and when integrated into soil samples, the impact of severe cold temperatures, characteristic of Martian conditions, was evaluated. In particular, the study focused on temperature conditions of -18 degrees Celsius and -80 degrees Celsius (**Figure 4.21**). Upon analysis, the data exhibited a notable degradation of cysteine when spiked into soil samples under both temperature conditions. However, the rate of degradation at -80 degrees Celsius was found to be lower than that at -18 degrees Celsius. This could suggest a potential influence of temperature on the rate of cysteine degradation, with colder conditions possibly slowing the degradation process.

Notably, when cysteine spiked into KGa-1b, the results took an intriguing turn. The cysteine demonstrated substantial stability at -80 degrees Celsius. This significant finding indicates the potential survivability of such molecules in the extreme cold conditions of Mars, specifically in the context of KGa-1b simulant. These results contribute valuable insights into the potential

behavior of biomolecules such as cysteine under Martian-like conditions. The findings further underline the importance of considering the specific environmental context, including soil composition and temperature, in the detection and interpretation of potential biomarkers in extraterrestrial life search missions.

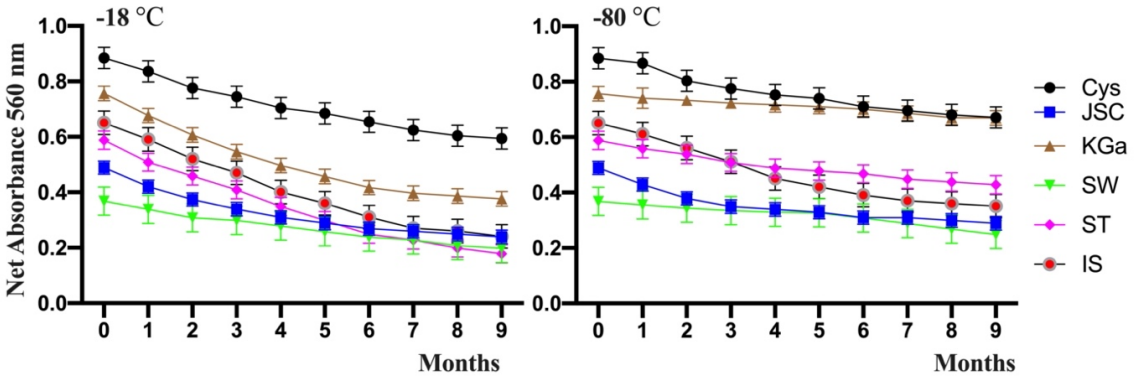


Figure 4.21. Thermal stability of cysteine in extreme cold conditions. The figure presents the resilience of both isolated cysteine and cysteine spiked-soil samples under temperatures of -18°C and -80°C, representative of Mars' harsh cold climate. Data represent average±SD (N=3).

4.3.3 Development of a method for the quantification of lipids on soil matrices

Initially, Krill was prepared in 1 mg/ml stock and diluted to 0.1 mg/ml in chloroform and dried in either glass vials or eppendorfs. Then 100 µl H_2SO_4 were added in to dissolve the krill and react with vanillin reagent (**Figure 4.22**). In this case, sulfuric acids did not interfere the absorbance in plastic eppendorfs. The standard curves from glass vials and eppendorfs were similar and liner.

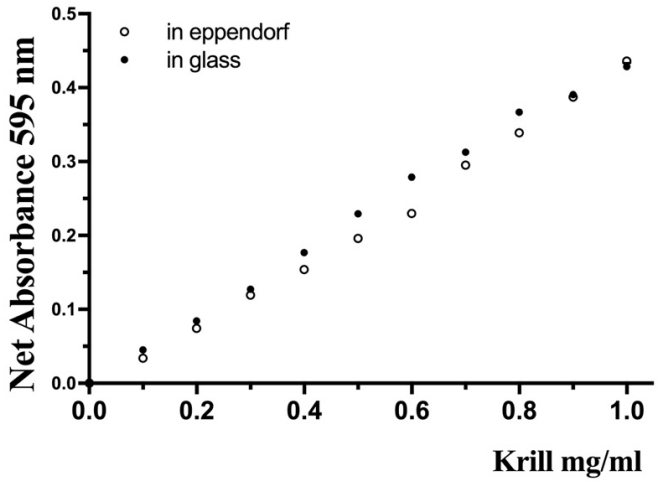


Figure 4.22. The impact of using glass material or plastic material on vanillin assay. Data represent average±SD (N=3).

Then the impact of heating was introduced to explore the sensitivity of this assay. 0.5 mg/ml Krill was dried in either glass vials or plastic eppendorfs. Then sulfuric acid was added to dissolve the krill and incubated at 96 ℃ in water bath for 10 minutes (the control was incubated at room temperature), and then react with vanillin reagent (**Figure 4.23**). The result indicated that heating could improve the sensitivity of vanillin assay which showed a higher absorbance compared with the control without heating. Furthermore, it is better to use glass ware to perform this assay as the absorbance of controls from the plastic eppendorfs was higher than others which could interfere the final result.

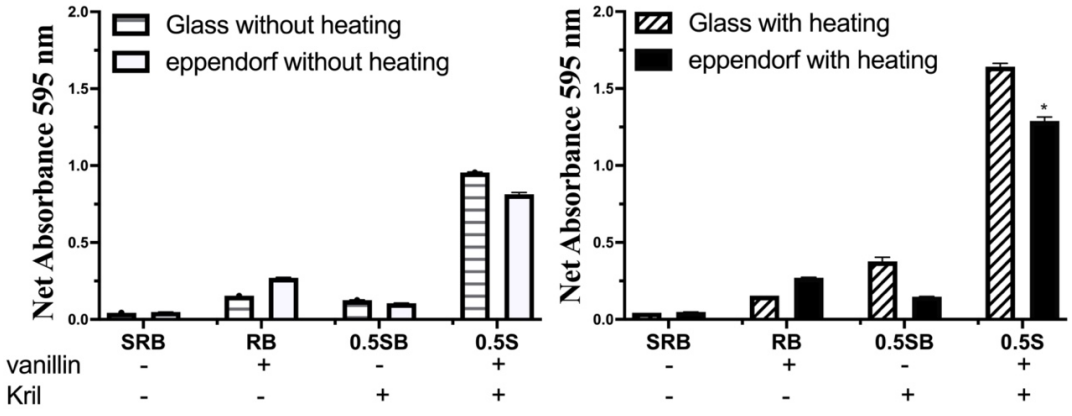


Figure 4.23. The impact of heating on vanillin assay with using glass and plastic material. Data represent average±SD (N=3). *Statistically significant by Student's *t*-test.

Having explored the best incubation condition, a series of different concentrations (from 1 to 10 μg) of krill was measured as the standard curve of the assay (**Figure 4.24**).

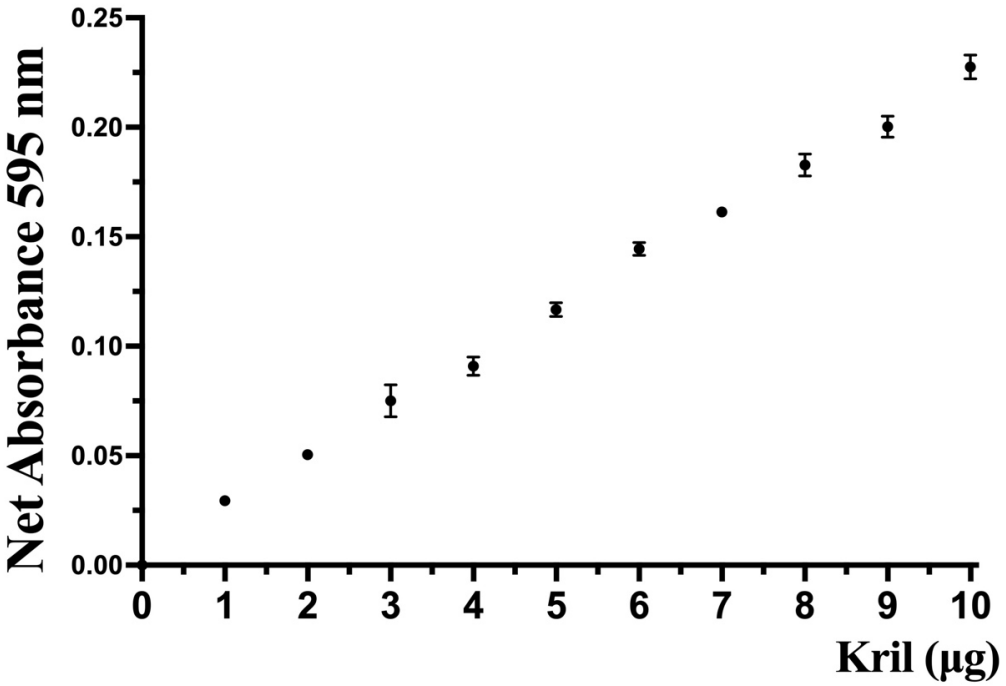


Figure 4.24. Linear standard curve of krill from 1 to 10 μg in 200 μl sample volume. Data represent average±SD (N=3).

Following the recovery of the Krill from the spiked soil was explored. Krill-spiked soil simulants were extracted in chloroform and dried in glass for the vanillin assay. They were also compared with the same concentration of pure Krill solution dried in glass (**Figure 4.25**). 20 μg Krill was spiked in 50 mg soil simulants and extracted in 1 ml chloroform (in total). The result showed the recovery of spiked-Krill from soil simulants was from 79.6% to 83.7% among in four times extractions (**Figure 4.25**). With the increase of the number of extractions, the absorbance of the control group (soil simulants without spiking krill) also increased which resulted in the lower net absorbance for the krill-spiked soil result.

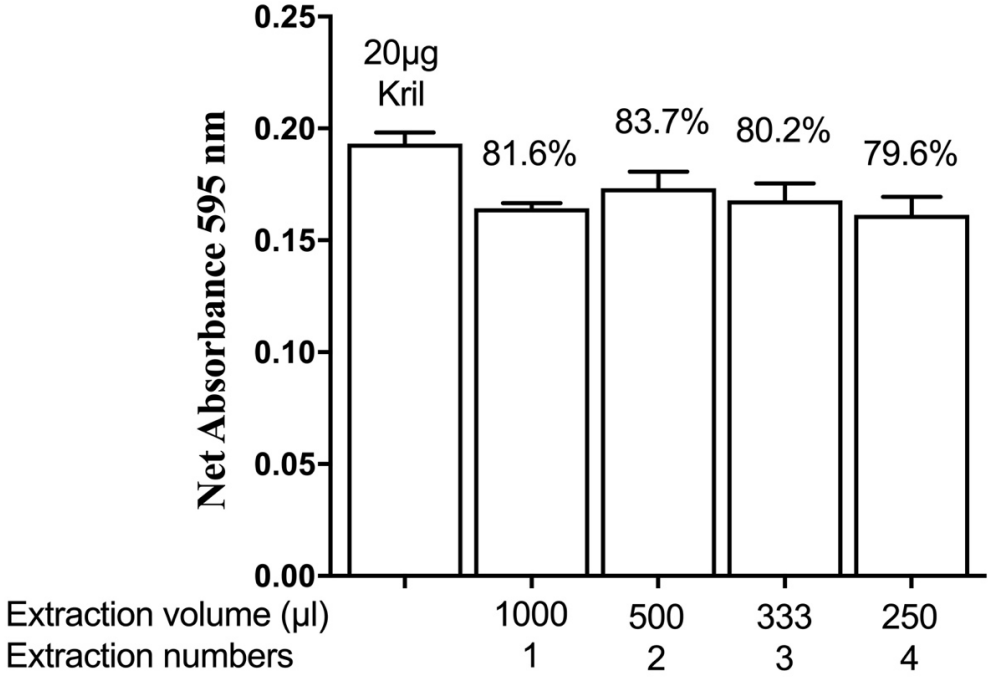


Figure 4.25. The recovery of krill lipid from spiked soil simulants. Data represent average±SD (N=3).

Then the krill was spiked in the soil simulants with a series of concentration and tested by using vanillin assay **(Figure 4.26)**. The impact of UV radiation was also explored in this krill-spiked soil matrix as UV radiation on the surface of Mars would result in adverse conditions for the existence of metabolites. The spiked soil matrix was exposed to UV radiation for up to 120 minutes **(Figure 4.27)**. This result indicated that UV radiation caused a significant decrease of the signal detected (after two hours, the signal decreased 26.9% compared with the spiked soil without UV radiation), which could be attributed to the decomposition of krill lipid spiked on the soil matrix.

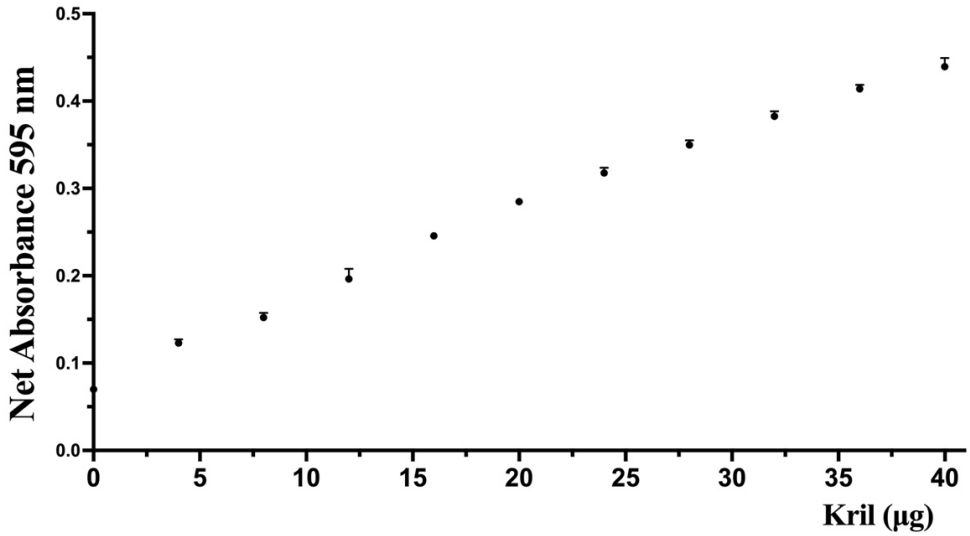


Figure 4.26. Quantification of Krill lipids spiked on the soil matrix. Data represent average±SD (N=3).

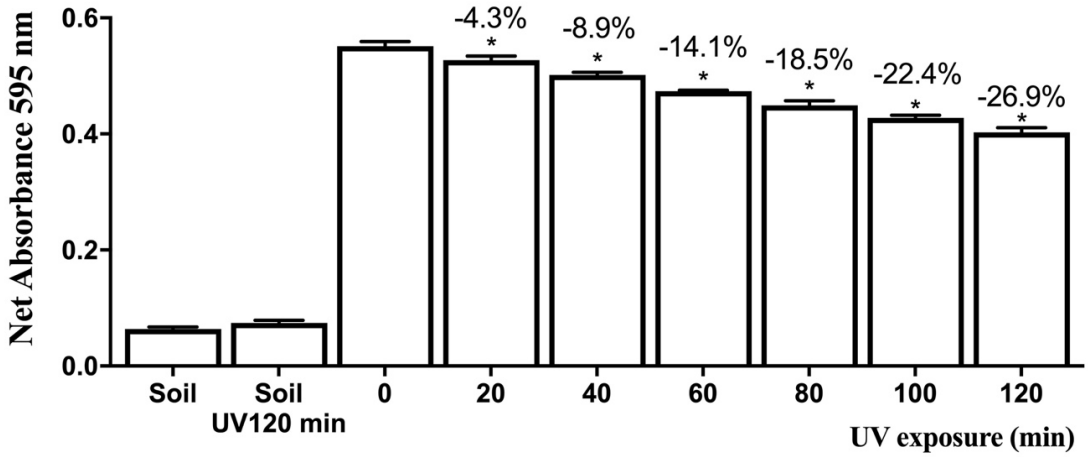


Figure 4.27. The impact of UV radiation on Krill-spiked soil matrix. Data represent average±SD (N=3). *Statistically significant by Student's *t*-test.

Due to the abundance of clay was found in Gale Crater on Mars (McLennan et al., 2014), several clay samples were also introduced in this research to explore the recovery of krilll and the impact of UV radiation on it. There were four different clays used in this research: Kaolinite (KGa-1b), Na-rich Montmorillonite (SWy-3), Montmorillonite (STx-1b) and Illite-smectite mixed layer (ISCz-1). All clay samples (50 mg) were spiked with 100 µg krill and extracted in chloroform, then 50 µl supernatants were dried in glass vials (25 µg krill in the glass) and reacted with vanillin assay **(Figure 4.28)**. JSC soil simulants were treated as the same way as the control to compare the impact of UV radiation, while krill without spiking soil was for the comparison of recovery. The result indicated that soil/clays could provide to some extent

protection from UV radiation, except ISCz-1, which showed the similar decrease of absorbance signal to no soil spiking samples. There is no significant difference of the protection between JSC soil simulants and clay samples. Among the soil/clay samples, the JSC provided a higher recovery (81.51%) and the recoveries for other clays were varied from 62.49% to 77.75%. The reason for causing the difference in recovery may be due to the physical adsorption and surface chemical activity of the clays.

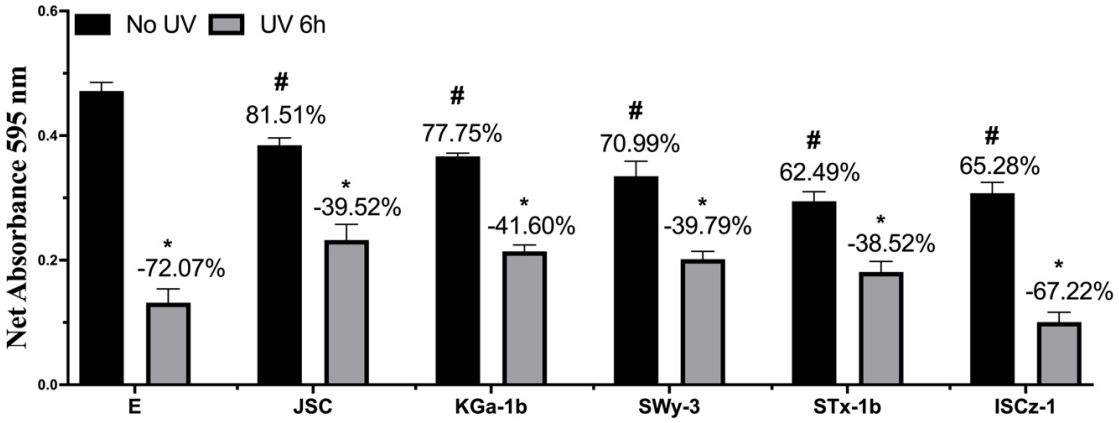


Figure 4.28. The recovery and impact of UV radiation on krill-spiked soil matrix/clays. Data represent average±SD (N=3). E: no spiking soil/clays. Statistically significant by two-way ANOVA corrected with post-hoc Turkey test. *Impact of UV radiation on samples, # recovery of the krill signal from soil/clay.

The stability of Krill lipids was also explored, both in isolation and when embedded within soil samples, under intense cold conditions reflective of the Martian environment. The temperature regimes concentrated on were -18 degrees Celsius and -80 degrees Celsius, to represent a range of conceivable Martian conditions **(Figure 4.29)**.

Upon analysis, a noteworthy degradation pattern was observed in krill lipids that were spiked into soil samples under both temperature conditions. Intriguingly, the degradation rate at -80 degrees Celsius was found to be slower than the rate observed at -18 degrees Celsius. This observation intimates a potential temperature-dependent modulation in the rate of lipid degradation. More specifically, it suggests that colder conditions might induce a deceleration in the degradation process.

An examination of the stability of krill lipids within these temperature contexts is crucial, given that lipids are integral to cellular structure and function. Understanding their potential preservation or degradation in extreme conditions contributes valuable insights to the field of astrobiology.

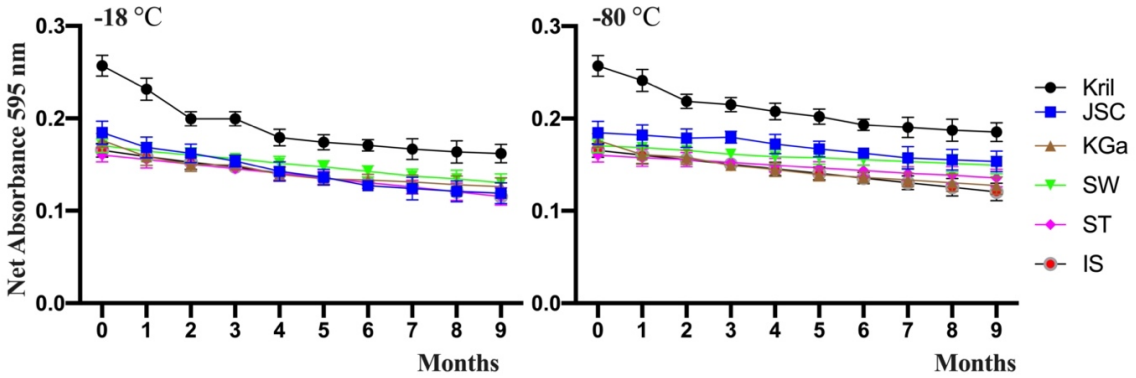


Figure 4.29. Thermal stability of krill in extreme cold conditions. The figure presents the resilience of both isolated krill and krill spiked-soil samples under temperatures of -18°C and -80°C, representative of Mars' harsh cold climate. Data represent average±SD (N=3).

4.3.4 Spiking, extraction and detection of amino acids on the Martian soil simulant

The presence and recovery of amino acids spiked into soil matrices was assessed by using LCMS. This is for preliminary earth-based studies to understand the behaviour, stability, and detectability of target molecules under simulated Martian conditions. These comprehensive analyses could provide detailed insights that will guide the selection and design of appropriate simplified instruments for actual space missions. In this method, soil samples were spiked with a 20 mM amino acid mixture. The extracts from these spiked soil matrices were reconstituted in 300 µl of 95% LC-MS grade water and 5% LC-MS grade methanol. After vigorous vortexing and centrifugation, the clear supernatant was isolated and analysed using HPLC and mass spectrometry.

Two solvents were used to extract the spiked soils in this experiment: 100% LC-MS grade water and a mixture of 80% LC-MS grade methanol with 20% LC-MS grade water **(Figure 4.30)**. The results indicated that water, as an extraction solvent, yielded a superior recovery of amino acids from the spiked soil matrices. This finding underscores the utility of a straightforward water-based extraction method for isolating amino acids from soil simulants.

Furthermore, we were able to detect all the amino acids from the spiked soil samples, underscoring the capability of HPLC and mass spectrometry as robust and sensitive tools for the detection of potential life-signalling amino acids in Martian soil simulants. This line of investigation, hence, offers promising avenues for future astrobiological studies, especially in the context of the search for biochemical evidence of life beyond Earth.

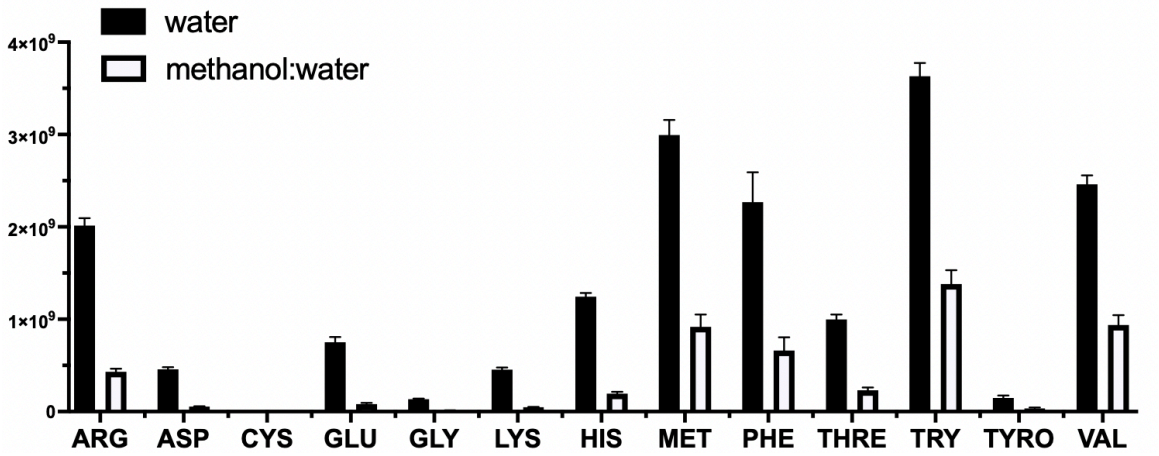


Figure 4.30. The detection of amino acids by using LC-MS. Data represent average±SD (N=5).

The influence of UV radiation on the amino acids spiked into the soil matrices was explored by employing Ultra-Performance Liquid Chromatography with Fluorescence (UPLC-FLR). For this, soil samples were spiked with a 2 mM amino acid mixture, half of which were exposed to UV radiation for six hours. The extraction of amino acids was quantified post-specific labelling with the fluorescence dye AccQ-TagTM (Waters). Subsequently, the derivatives were separated via reversed phase chromatography on an Acquity BEH C18 column (150 mm × 2.1 mm, 1.7 µm, Waters), linked to an Acquity H-class UPLC system and quantified through fluorescence detection (Acquity FLR detector, Waters) **(Figure 4.31)**. The result demonstrated a decrease in all amino acids following UV exposure. This reduction in signal detection is a testament to the adverse effects UV radiation can inflict upon amino acids present in Martian soil simulants. The UV radiation, owing to its high-energy photons, can instigate changes in the molecular structures of amino acids, leading to their fragmentation or alteration. Consequently, these destructive processes likely contribute to the recorded decrease in signal detection, as the abundance of original form amino acids is compromised. Thus, the results underscore the urgency in developing robust and sensitive methodologies for extracting and detecting biological markers from harsh environmental conditions. They also emphasise the necessity to accommodate potential UV-induced biases when interpreting results from UV-exposed amino acid analyses.

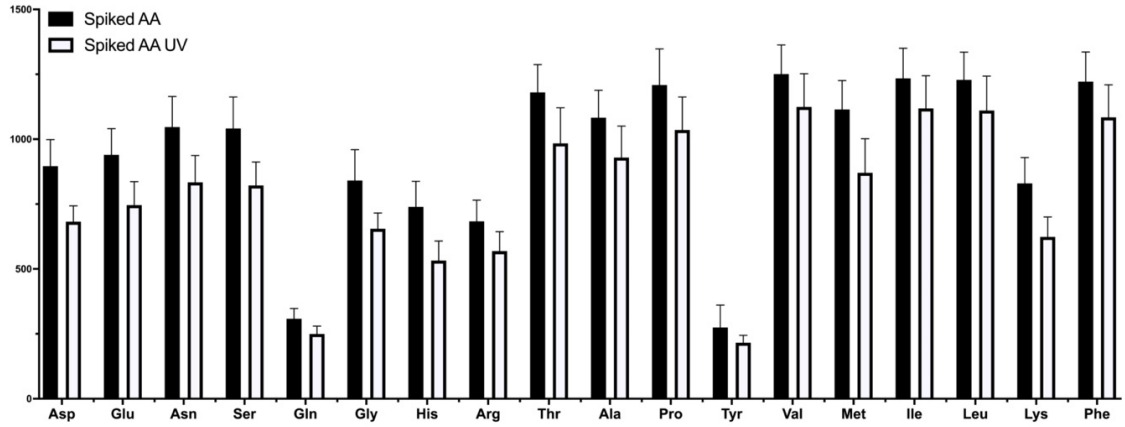


Figure 4.31. The influence of UV radiation on the amino acids spiked into the soil matrices by employing Ultra-Performance Liquid Chromatography with Fluorescence (UPLC-FLR). Data represent average±SD (N=5).

4.3.5 Spiking, extraction and detection of lipids on the Martian soil simulant

Following, the undertaken research evaluated the degradation behaviour of FAMEs under UV exposure using GC-MS. The findings presented a compelling insight into lipid stability, underlining that lipids with longer chain lengths are more prone to degradation under UV irradiation **(Figure 4.32)**.

A thorough investigation identified that FAMEs with extended acyl chains, which typify complex lipid structures, were distinctly vulnerable to UV-induced degradation. This predilection could be a consequence of their amplified structural complexity and a larger number of reactive sites. These factors offer increased opportunities for UV radiation to prompt degradation processes. These discoveries prompt significant implications for the examination of residual lipids in environments subjected to intense UV radiation levels, akin to the Martian surface. In such conditions, there may be a preferential preservation of shorter chain lipids, resulting in a skewed representation of the lipid distribution as detected through this methodological approach. Thus, consideration of this UV-induced bias is imperative when interpreting outcomes from ensuing lipid analyses conducted on Martian soil simulants.

In sum, the findings offer pivotal insights into the varying stability of lipid structures under UV exposure. This augments our comprehension of lipid persistence in extra-terrestrial settings, and consequently guides our quest for potential lipid-based biomarkers on Mars.

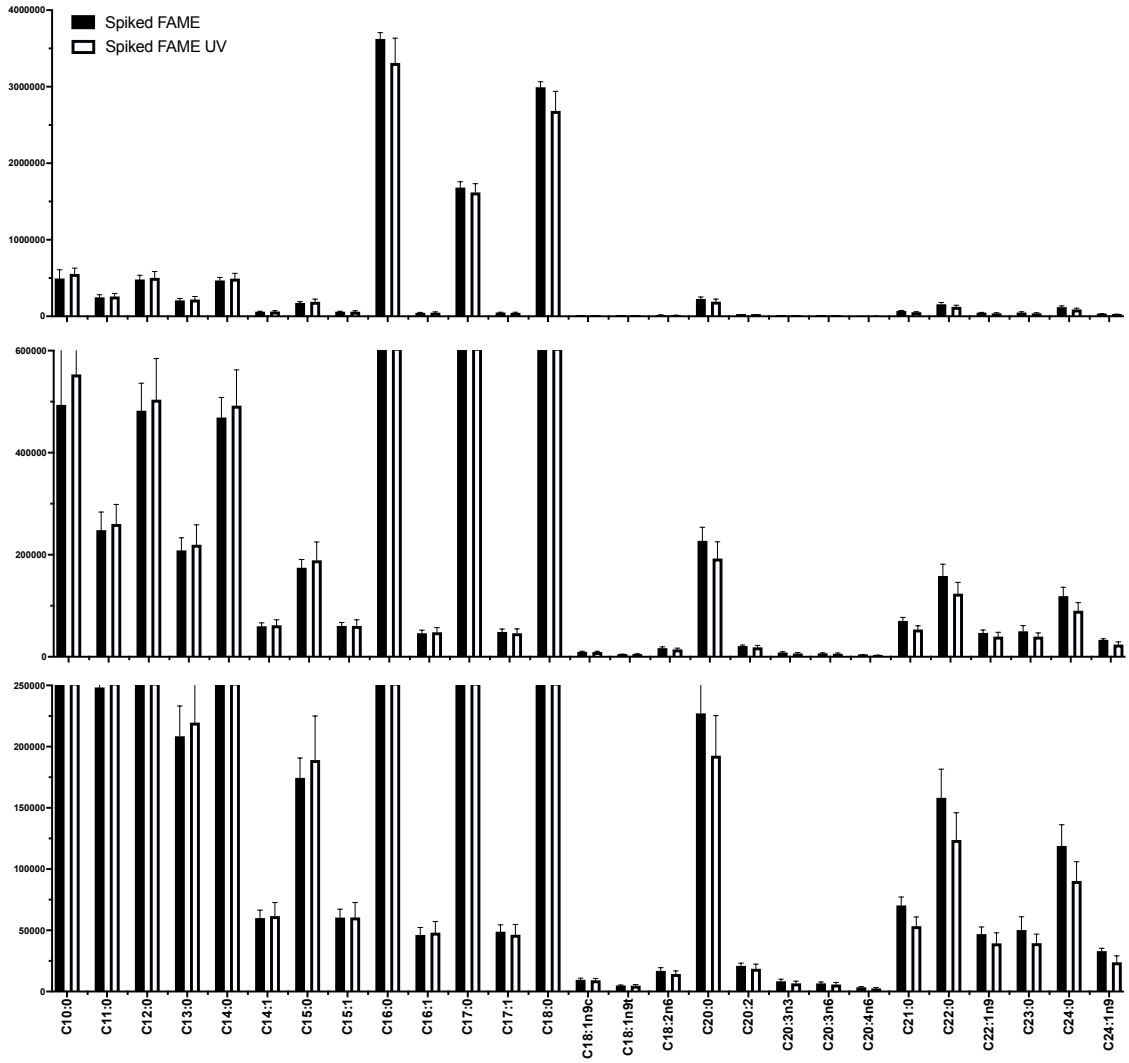


Figure 4.32. The detection of FAMEs under UV exposure using GC-MS. Data represent average±SD (N=5).

4.4 Discussion

In this chapter, we developed and optimised a series of methods to detect reduced thiols (glutathione and cysteine), complex lipids (krill oil) and amino acids. For the detection of glutathione, it was based on the reaction of DTP with glutathione, and it can detect the glutathione as low as 2 μM. While for Cysteine, it was based on the reaction with ninhydrin and the minimum detectable concentration was 5 μM. Lipids detection was based on the vanillin assay and the minimum detectable concentration for krill was 1 μg in 200 μl sample volume. Finally, a mixture of amino acids and lipids were spiked on JSC soil simulants respectively and analysed by Liquid chromatography-mass spectrometry (LC-MS) and gas chromatography-mass spectrometry (GC-MS). These methods could offer detailed characterization which was crucial for the primary identification of potential biosignatures and understanding their behaviour under various conditions.

In the detection of thiols, we optimised the approach of spiking, extracting, and recovering for glutathione and cysteine. The solvent for dissolving glutathione was explored by using methanol and acetic acid and the result indicated that methanol is the better option as the solvent. The reason could be explained that glutathione has low stability in water (Yesiltepe et al., 2021). While for cysteine, it also showed a good solubility and stability in methanol (Han et al., 2020). After extracting the glutathione-spiked soil simulants in water, the recovery can be up to 92.8% after four times extraction. However, for the recovery of cysteine, we tried to use different buffer for the extraction, the best option was to use 100 mM HCl to extract cysteine and the recovery is 40.4%. This is because the acidic condition can minimize thiol oxidation by suppressing the ionization of thiols to thiol anions. Other techniques for the detection of total thiols such as using HPLC to quantify 5-thio-2-nitrobenzoic acid (TNB) which can be generated through the reaction of thiols with 5,5′-dithiobis (2-nitrobenzoic acid) (Chen et al., 2008). It was demonstrated with OVCAR-3 cells and required a high standard of purity for solutions and may not be suitable for in situ analysis on the surface of Mars. Our methods for detecting thiols are straightforward and need less steps to perform. For the detection of glutathione, it only needs to prepare the 0.75 mM DTP stock in advance and react with the sample extraction. While for the detection of cysteine, it needs to prepare 60 mM ninhydrin in advance and incubate with the sample extraction for 10 minutes at 100 °C. After that, the absorbance can be measured to quantify glutathione and cysteine. These methods are sensitive and specific, which can detect the thiols in a short time. Although it needs to make the

extraction for the soil samples in advance, these methods can provide a quick and economic way as a first attempt for detection of thiols in situ.

For the detection of lipids, krill lipids could potentially be used as biomarkers for the detection of metabolites on Mars because they are a type of biomolecule that is particularly resistant to degradation, making them a good candidate for long-term preservation in the harsh Martian environment (Yesiltepe et al., 2021). Krill are small, shrimp-like crustaceans that are abundant in many of the oceans in the world, including the Southern Ocean surrounding Antarctica (Everson, 2000). Krill lipids are composed of a variety of different types of fatty acids (FA) and other polar lipids, such as phospholipids (PL), and they are known to be highly resistant to oxidative and thermal degradation (Xie et al., 2017, Bustos et al., 2003). This stability makes krill lipids a good candidate for use as biomarkers in the search for evidence of life on Mars or other planetary bodies. In this chapter, we optimized the vanillin assay for the detection of lipids including neutral lipids, phospholipids, and sphingolipids, in biological samples (Mishra et al., 2014).

The traditional vanillin assay based on the sulfo-phospho-vanillin (SPV) reaction which required that by mixing the lipid extract with sulfuric acid and heating the mixture, then adding the phospho-vanillin reagent to activate the reaction between vanillin and lipids, forming a characteristic chromophore (Frings and Dunn, 1970). Our method explored the impact of plastic on detecting lipids and also validated the necessary of heating for the reaction. After spiking lipids on JSC soil simulants as well as clays, a good recovery was observed in JSC, Kaolinite and Na-rich Montmorillonite using the chloroform for the extraction, which the rate of recovery was over 70%. The reason for the lower recovery from Montmorillonite and Illite-smectite mixed layer clays maybe because lipids may adsorb onto the surface of the clay particles, reducing the amount of lipid available for extraction, as the high surface area of these two clays (Meyers and Quinn, 1974). In the intricate structure of clay minerals, particularly layered varieties, lipids could potentially be entrapped and shielded within these layers, effectively reducing the amount available for extraction. It is imperative to understand the dynamics of clay minerals in order to gain insights into the lipid recovery challenges observed. The potential for lipids to be protected within the layers of clay minerals is a critical aspect that may explain the lower recovery rates. The layered structure of some clay minerals, especially Montmorillonite and Illite-smectite mixed layer clays, could facilitate the attraction and entrapment of lipids, thereby rendering them less accessible during the extraction process. To overcome these challenges and improve the recovery of spiked lipids, it may need to use some

complementary techniques, such as ultrasonication or the addition of surfactants. In addition, it may be necessary to use multiple extraction techniques, or to extract the lipids in multiple steps, to ensure complete extraction of the lipids from the sample. By exploring the impact of UV radiation on lipids, with the increase of the UV exposure time, the lipids showed the gradual degradation. When lipids were exposed to UV radiation, photochemical reactions can break down the molecular structure of lipids, leading to the formation of smaller, more oxidized, and less biologically relevant compounds which cannot be detected by vanillin assay (Bacellar and Baptista, 2019). In addition, UV radiation can also directly oxidize lipids, leading to the formation of peroxides, aldehydes, and other oxidized compounds that are not representative of the original lipid structure (Ayala et al., 2014). These oxidized compounds can interfere with the detection of lipids as biomarkers and make it more difficult to confirm the existence of extraterrestrial life based on the detection of lipids.

Amino acids, as the building blocks of proteins, have been considered as potential metabolites for the detection of extraterrestrial life. Unlike lipids, amino acids are less prone to degradation under UV exposure, which is an important factor to take into consideration when searching for evidence of life in extraterrestrial environments. The presence of amino acids can indicate the existence of life, as they are the fundamental building blocks of proteins and play a crucial role in biological processes. In addition, the chirality, or handedness, of amino acids is a key characteristic that is believed to be related to the origin of life (Bailey, 2000). The detection of specific enantiomers, or stereoisomers, of amino acids could provide further evidence of life as the specific enantiomeric composition of amino acids is unique to living systems (Burton and Berger, 2018). The use of amino acids as metabolites for the detection of extraterrestrial life is an active area of research, and recent studies have reported the detection of amino acids in various extraterrestrial environments, including comets, meteorites, and Mars. By using LC-MS water for the extraction from amino acids-spiked soil simulants, and subsequent analysis via HPLC and mass spectrometry not only demonstrated a simple and efficient extraction method, but also the capability to detect a comprehensive range of amino acids from soil simulants. The efficiency and simplicity of the method developed here expand upon existing methods used for amino acid detection, often marked by a high level of complexity and requirement for specialized equipment.

The successful detection of a wide range of amino acids in soil simulants under simulated Martian conditions has significant implications for astrobiology and the search for potential life beyond Earth. These findings lend support to the hypothesis that life, as we understand it,

could survive in similar conditions, or at least, leave detectable traces of its biochemical constituents. This is particularly relevant to Mars, where recent missions have focused on the detection of organic molecules such as amino acids.

Nonetheless, there are many factors yet to be considered. Future research should aim to understand how these amino acids would degrade under varying environmental conditions, including the presence of perchlorates, a class of chemicals known to be present in Martian soils, which are thought to be harmful to organic molecules. Moreover, the study of chirality, the 'handedness' of amino acids, could be a critical area of future study. Terrestrial life utilizes left-handed amino acids almost exclusively, and a discovery of a chiral imbalance on Mars or elsewhere could provide compelling evidence of extraterrestrial life.

In response to the valid observations raised, it's essential to contextualize the utilization of LCMS and GC-MS in this study as foundational and preparatory, tailored for a comprehensive earth-bound analysis. We fully acknowledge the intrinsic constraints associated with space missions, notably the imperatives of instrumental compactness and operational simplicity. The detailed analytical insights gleaned from the employment of LCMS and GC-MS are instrumental in offering nuanced understandings of the behavior and detection of target biomolecules under simulated Martian environments. These insights are anticipated to be cardinal in steering the innovation, adaptation, and miniaturization of analytical technologies for their suitability in extraterrestrial explorations. Ongoing technological advancements are promising, with the emergence of compact, efficient, and automated versions of these analytical tools that are congruent with the logistical and operational exigencies of space missions. Thus, while we employed intricate methodologies for an in-depth analysis in this instance, the eventual objective remains anchored in aligning such sophisticated analytical processes with the practical realities of space exploration, ensuring data integrity, and operational feasibility are mutually inclusive.

In conclusion, the research presents a promising step towards the reliable detection of amino acids, a critical factor in our search for signs of life beyond Earth. The continued optimization and exploration of this method will prove invaluable in our quest to understand the universe and our place within it.

4.5 Conclusion

The research outlined in this chapter revolved around the development and refinement of uncomplicated biochemical methodologies to detect various metabolites that might serve as indicators of life. The metabolites of focus, thiols, play a vital role in mitigating oxidative stress, which is integral to maintaining normal cellular metabolism and physiological function, essential aspects of life. The thiols investigated in this research were glutathione and cysteine. The experimental design endeavoured to ascertain the most conducive spiking conditions and recovery rates. Methanol emerged as the optimal solvent for spiking and expeditious drying in a speedvac. The spiked soil was subsequently extracted in water and assayed. For glutathione, the recovery rate was impressive, exceeding 90%, while for cysteine, the rate hovered around 40%. To emulate Martian conditions, all spiked soils underwent UV radiation exposure. The findings underscored that UV radiation triggers thiols degradation, suggesting that these thiols may persist beneath the Martian surface.

Lipids were another focal point, viewed as complex metabolite compounds potentially signalling life. The methodological exploration and troubleshooting of lipid detection, including spiking and extraction, indicated chloroform as the optimal solvent for spiking and vanillin assay for lipid quantification. The effects of UV radiation and low temperatures were evaluated, and various clays were introduced to test their protective capabilities against UV radiation. Results demonstrated that both soil simulants and clays can safeguard lipids from UV radiation. Furthermore, GCMS results indicated that FAMEs with extended acyl chains, emblematic of intricate lipid structures, were notably susceptible to UV-induced degradation.

Integral to this research was the exploration of clays' role in the preservation of thiols and lipids. The nuanced interaction between thiols/lipids and clays, particularly the protective attributes of clays against UV radiation, has been spotlighted. The embedding of thiols and lipids within clay layers emerged as a significant facet, presenting a barrier to UV radiation and aiding in the preservation of these essential

biomolecules.

Lastly, amino acids, critical life components, were incorporated in this research. Amino acids-spiked soil simulants were extracted in LC-MS water and analysed via HPLC and mass spectrometry. This approach showcased a simple extraction method and the potential to detect a comprehensive range of amino acids from soil simulants. The study, in conclusion, advances our understanding of the potential biomarkers for life, strengthening the foundation for future exploration of life beyond Earth.

4.6 Reference

AOYAMA, K. & NAKAKI, T. 2015. Glutathione in cellular redox homeostasis: association with the excitatory amino acid carrier 1 (EAAC1). *Molecules,* 20**,** 8742-8758.

AYALA, A., MUNOZ, M. F. & ARGUELLES, S. 2014. Lipid peroxidation: production, metabolism, and signaling mechanisms of malondialdehyde and 4-hydroxy-2-nonenal. *Oxid Med Cell Longev,* 2014**,** 360438.

BACELLAR, I. O. L. & BAPTISTA, M. S. 2019. Mechanisms of Photosensitized Lipid Oxidation and Membrane Permeabilization. *ACS Omega,* 4**,** 21636-21646.

BAILEY, J. 2000. Chirality and the origin of life. *Acta Astronautica,* 46**,** 627-631.

BAPAT, N. V. & RAJAMANI, S. 2023. Distinguishing Biotic vs. Abiotic Origins of 'Bio'signatures: Clues from Messy Prebiotic Chemistry for Detection of Life in the Universe. *Life,* 13**,** 766.

BHATTARU, S. A. 2018. *Design, testing, and validation of the search for Extra-Terrestrial Genomes instrument.* Massachusetts Institute of Technology.

BORNMAN, J. F., BARNES, P. W., ROBSON, T. M., ROBINSON, S. A., JANSEN, M. A., BALLARÉ, C. L. & FLINT, S. D. 2019. Linkages between stratospheric ozone, UV radiation and climate change and their implications for terrestrial ecosystems. *Photochemical & Photobiological Sciences,* 18**,** 681-716.

BURRI, L., HOEM, N., BANNI, S. & BERGE, K. 2012. Marine omega-3 phospholipids: metabolism and biological activities. *International journal of molecular sciences,* 13**,** 15401-15419.

BURTON, A. S. & BERGER, E. L. 2018. Insights into Abiotically-Generated Amino Acid Enantiomeric Excesses Found in Meteorites. *Life (Basel),* 8.

BUSTOS, R., ROMO, L., YÁÑEZ, K., DÍAZ, G. & ROMO, C. 2003. Oxidative stability of carotenoid pigments and polyunsaturated fatty acids in microparticulate diets containing krill oil for nutrition of marine fish larvae. *Journal of Food Engineering,* 56**,** 289-293.

CHEN, W., ZHAO, Y., SEEFELDT, T. & GUAN, X. 2008. Determination of thiols and

disulfides via HPLC quantification of 5-thio-2-nitrobenzoic acid. *J Pharm Biomed Anal,* 48**,** 1375-80.

COEN, M., HOLMES, E., LINDON, J. C. & NICHOLSON, J. K. 2008. NMR-based metabolic profiling and metabonomic approaches to problems in molecular toxicology. *Chem Res Toxicol,* 21**,** 9-27.

COMBS, J. A. & DENICOLA, G. M. 2019. The non-essential amino acid cysteine becomes essential for tumor proliferation and survival. *Cancers,* 11**,** 678.

CORNISH-BOWDEN, A. 2004. *The Pursuit of Perfection: Aspects of Biochemical Evolution*, Oxford University Press, 2004.

DEBERARDINIS, R. J. & THOMPSON, C. B. 2012. Cellular metabolism and disease: what do metabolic outliers teach us? *Cell,* 148**,** 1132-1144.

DUVE, C. D. 2005. *Singularities: Landmarks on the Pathways of Life* Cambridge University Press.

EMBL-EBI. 2020. *What is metabolomics?* [Online]. Available: https://www.ebi.ac.uk/training/online/course/introduction-metabolomics/what-metabolomics [Accessed May 16th 2020].

EVERSON, I. 2000. Management of Krill Fisheries in Canadian Waters. *Krill.*

EXPLORINGORIGINS. 2020. *THE RNA WORLD HYPOTHESIS* [Online]. Available: http://exploringorigins.org/ribozymes.html [Accessed May 5th 2020].

FANI, R. & FONDI, M. 2009. Origin and evolution of metabolic pathways. *Phys Life Rev,* 6**,** 23-52.

FELL, D. & CORNISH-BOWDEN, A. 1997. *Understanding the control of metabolism*, Portland press London.

FONDI, M., EMILIANI, G. & FANI, R. 2009. Origin and evolution of operons and metabolic pathways. *Res Microbiol,* 160**,** 502-12.

FORMAN, H. J., ZHANG, H. & RINNA, A. 2009. Glutathione: overview of its protective roles, measurement, and biosynthesis. *Molecular aspects of medicine,* 30**,** 1-12.

FRINGS, C. S. & DUNN, R. T. 1970. A colorimetric method for determination of total serum lipids based on the sulfo-phospho-vanillin reaction. *Am J Clin Pathol,* 53**,**

89-91.

FUSZ, S., EISENFUHR, A., SRIVATSAN, S. G., HECKEL, A. & FAMULOK, M. 2005. A ribozyme for the aldol reaction. *Chem Biol,* 12**,** 941-50.

GRANICK, S. 1957. Speculations on the origins and evolution of photosynthesis. *Ann N Y Acad Sci,* 69**,** 292-308.

GRANICK, S. 1965. Evolution of Heme and Chlorophyll. *Evolving Genes and Proteins.*

GRINTZALIS, K., PAPAPOSTOLOU, I., ZISIMOPOULOS, D., STAMATIOU, I. & GEORGIOU, C. D. 2014. Multiparametric protocol for the determination of thiol redox state in living matter. *Free Radic Biol Med,* 74**,** 85-98.

HAN, J., LIU, H., HU, S., QIU, J., YI, D., AN, M., GUO, Y., HUANG, H., HE, H. & WANG, P. 2020. Determination and Correlation of the Solubility of l-Cysteine in Several Pure and Binary Solvent Systems. *Journal of Chemical & Engineering Data,* 65**,** 2649-2658.

HELLESSEY, N., JOHNSON, R., ERICSON, J. A., NICHOLS, P. D., KAWAGUCHI, S., NICOL, S., HOEM, N. & VIRTUE, P. 2020. Antarctic krill lipid and fatty acid content variability is associated to satellite derived chlorophyll a and sea surface temperatures. *Scientific Reports,* 10**,** 6060.

HOROWITZ, N. H. 1945. On the Evolution of Biochemical Syntheses. *Proc Natl Acad Sci U S A,* 31**,** 153-7.

JENSEN, R. A. 1976. Enzyme recruitment in evolution of new function. *Annu Rev Microbiol,* 30**,** 409-25.

JUDGE, A. & DODD, M. S. 2020. Metabolism. *Essays Biochem,* 64**,** 607-647.

KANANI, H., CHRYSANTHOPOULOS, P. K. & KLAPA, M. I. 2008. Standardizing GC-MS metabolomics. *J Chromatogr B Analyt Technol Biomed Life Sci,* 871**,** 191-201.

KELLER, M. A., KAMPJUT, D., HARRISON, S. A. & RALSER, M. 2017. Sulfate radicals enable a non-enzymatic Krebs cycle precursor. *Nat Ecol Evol,* 1**,** 83.

KELLER, M. A., TURCHYN, A. V. & RALSER, M. 2014. Non-enzymatic glycolysis and pentose phosphate pathway-like reactions in a plausible Archean ocean. *Mol Syst Biol,* 10**,** 725.

LAZCANO, A. & MILLER, S. L. 1996. The Origin and Early Evolution of Life: Prebiotic Chemistry, the Pre-RNA World, and Time. *Cell,* 85**,** 793-798.

LEE, C., WEBER, J. M., RODRIGUEZ, L. E., SHEPPARD, R. Y., BARGE, L. M., BERGER, E. L. & BURTON, A. S. 2022. Chirality in organic and mineral systems: A review of reactivity and alteration processes relevant to prebiotic chemistry and life detection missions. *Symmetry,* 14**,** 460.

LOPEZ, M. J. & MOHIUDDIN, S. S. 2020. Biochemistry, essential amino acids.

MAHAFFY, P. 2007. Exploration of the Habitability of Mars: Development of Analytical Protocols for Measurement of Organic Carbon on the 2009 Mars Science Laboratory. *Space Science Reviews,* 135**,** 255-268.

MAIER, T. V. & SCHMITT-KOPPLIN, P. 2016. Capillary Electrophoresis in Metabolomics. *Methods Mol Biol,* 1483**,** 437-70.

MCLENNAN, S. M., ANDERSON, R. B., BELL, J. F., 3RD, BRIDGES, J. C., CALEF, F., 3RD, CAMPBELL, J. L., CLARK, B. C., CLEGG, S., CONRAD, P., COUSIN, A., DES MARAIS, D. J., DROMART, G., DYAR, M. D., EDGAR, L. A., EHLMANN, B. L., FABRE, C., FORNI, O., GASNAULT, O., GELLERT, R., GORDON, S., GRANT, J. A., GROTZINGER, J. P., GUPTA, S., HERKENHOFF, K. E., HUROWITZ, J. A., KING, P. L., LE MOUELIC, S., LESHIN, L. A., LEVEILLE, R., LEWIS, K. W., MANGOLD, N., MAURICE, S., MING, D. W., MORRIS, R. V., NACHON, M., NEWSOM, H. E., OLLILA, A. M., PERRETT, G. M., RICE, M. S., SCHMIDT, M. E., SCHWENZER, S. P., STACK, K., STOLPER, E. M., SUMNER, D. Y., TREIMAN, A. H., VANBOMMEL, S., VANIMAN, D. T., VASAVADA, A., WIENS, R. C., YINGST, R. A. & TEAM, M. S. L. S. 2014. Elemental geochemistry of sedimentary rocks at Yellowknife Bay, Gale crater, Mars. *Science,* 343**,** 1244734.

MEYERS, P. A. & QUINN, J. G. 1974. Organic matter on clay minerals and marine sediments — effect on adsorption of dissolved copper, phosphate, and lipids from saline solutions. *Chemical Geology,* 13**,** 63-68.

MISHRA, S. K., SUH, W. I., FAROOQ, W., MOON, M., SHRIVASTAV, A., PARK, M. S. & YANG, J. W. 2014. Rapid quantification of microalgal lipids in aqueous

medium by a simple colorimetric method. *Bioresour Technol,* 155**,** 330-3.

MORA, M. F., STOCKTON, A. M. & WILLIS, P. A. 2013. Analysis of thiols by microchip capillary electrophoresis for in situ planetary investigations. *Electrophoresis,* 34**,** 309-16.

MOROWITZ, H. J. 1992. *Beginnings of Cellular Life: Metabolism Recapitulates Biogenesis* Yale University Press.

NAVARRO-GONZALEZ, R., NAVARRO, K. F., DE LA ROSA, J., INIGUEZ, E., MOLINA, P., MIRANDA, L. D., MORALES, P., CIENFUEGOS, E., COLL, P., RAULIN, F., AMILS, R. & MCKAY, C. P. 2006. The limitations on organic detection in Mars-like soils by thermal volatilization-gas chromatography-MS and their implications for the Viking results. *Proc Natl Acad Sci U S A,* 103**,** 16089-94.

PERETÓ, J. 2011. *Origin and evolution of metabolisms* [Online]. Available: https://www.uv.es/genevol/pdfs/2011_proofs_c18_p270-288.pdf [Accessed May 16th 2020].

PIZZARELLO, S. 2006. The chemistry of life's origin: a carbonaceous meteorite perspective. *Accounts of Chemical Research,* 39**,** 231-237.

PLUMB, R. S., STUMPF, C. L., GORENSTEIN, M. V., CASTRO-PEREZ, J. M., DEAR, G. J., ANTHONY, M., SWEATMAN, B. C., CONNOR, S. C. & HASELDEN, J. N. 2002. Metabonomics: the use of electrospray mass spectrometry coupled to reversed-phase liquid chromatography shows potential for the screening of rat urine in drug development. *Rapid Commun Mass Spectrom,* 16**,** 1991-6.

PROSS, A. 2016. *What is life?: How chemistry becomes biology*, Oxford University Press.

PULLEN, J. & SAEED, K. 2012. An overview of biodiesel oxidation stability. *Renewable and Sustainable Energy Reviews,* 16**,** 5924-5950.

RALSER, M. 2014. The RNA world and the origin of metabolic enzymes. *Biochem Soc Trans,* 42**,** 985-8.

RUMMEL, J. D. 2001. Planetary exploration in the time of astrobiology: protecting

against biological contamination. *Proceedings of the National Academy of Sciences,* 98**,** 2128-2131.

SUMMONS, R. E., ALBRECHT, P., MCDONALD, G. & MOLDOWAN, J. M. 2008. Molecular Biosignatures. *Strategies of Life Detection.*

THEODORIDIS, G., GIKA, H. G. & WILSON, I. D. 2011. Mass spectrometry-based holistic analytical approaches for metabolite profiling in systems biology studies. *Mass Spectrom Rev,* 30**,** 884-906.

THEODORIDIS, G. A., GIKA, H. G., WANT, E. J. & WILSON, I. D. 2012. Liquid chromatography-mass spectrometry based global metabolite profiling: a review. *Anal Chim Acta,* 711**,** 7-16.

TOMÁS-BARBERÁN, F. A. 1995. Capillary electrophoresis: A new technique in the analysis of plant secondary metabolites. *Phytochemical Analysis,* 6**,** 177-192.

TOYO'OKA, T. 2009. Recent advances in separation and detection methods for thiol compounds in biological samples. *J Chromatogr B Analyt Technol Biomed Life Sci,* 877**,** 3318-30.

VEMURI, G. N. & ARISTIDOU, A. A. 2005. Metabolic engineering in the-omics era: elucidating and modulating regulatory networks. *Microbiology and Molecular Biology Reviews,* 69**,** 197-216.

WESTALL, F. & CAVALAZZI, B. 2011. Biosignatures in rocks. Springer-Verlag.

WOLFENDER, J.-L., MARTI, G., THOMAS, A. & BERTRAND, S. 2015. Current approaches and challenges for the metabolite profiling of complex natural extracts. *Journal of Chromatography A,* 1382**,** 136-164.

XIE, D., JIN, J., SUN, J., LIANG, L., WANG, X., ZHANG, W., WANG, X. & JIN, Q. 2017. Comparison of solvents for extraction of krill oil from krill meal: Lipid yield, phospholipids content, fatty acids composition and minor components. *Food Chem,* 233**,** 434-441.

XU, Z., HARVEY, K., PAVLINA, T., DUTOT, G., ZALOGA, G. & SIDDIQUI, R. 2010. An improved method for determining medium-and long-chain FAMEs using gas chromatography. *Lipids,* 45**,** 199-208.

YANG, Y., A, M. P., HOFLER, C., POSCHET, G., WIRTZ, M., HELL, R. & SOURJIK,

V. 2015. Relation between chemotaxis and consumption of amino acids in bacteria. *Mol Microbiol,* 96**,** 1272-82.

YČAS, M. 1974. On earlier states of the biochemical system. *Journal of Theoretical Biology,* 44**,** 145-160.

YESILTEPE, O., GULER CELIK, E., GEYIK, C., GUMUS, Z. P., ODACI DEMIRKOL, D., COSKUNOL, H. & TIMUR, S. 2021. Preparation of glutathione loaded nanoemulsions and testing of hepatoprotective activity on THLE-2 cells. *Turk J Chem,* 45**,** 436-451.

ZHANG, W., HANKEMEIER, T. & RAMAUTAR, R. 2017. Next-generation capillary electrophoresis-mass spectrometry approaches in metabolomics. *Curr Opin Biotechnol,* 43**,** 1-7.

Chapter 5: A simple method for the detection of microbes on Martian analogue soils

Abstract

The possibility of existence of microbial life beyond Earth remains a central inquiry in the field of astrobiology. The premise that extra-terrestrial life, if present, is likely to be microbial, underscores the necessity for robust, sensitive, and specific validation methods for recognizing potential life-bearing habitats. This research delineates a pioneering method for detecting microbial entities in Martian soil simulants, achieved through meticulous spiking and recovery experiments. This study utilizes miniaturized technologies, facilitating the swift and sensitive examination of soil samples for microbial presence. The findings from this research propose that this methodology could serve as an essential instrument for pinpointing potential habitats harbouring microbial life on Mars and other celestial bodies. This approach contributes significantly to our quest for extra-terrestrial life, shedding light on new potential pathways for astrobiological research.

5.1 Introduction

5.1.1 The Evolution of microbial life on Earth

It has been reported that through fossil evidence and new molecular clock analyses, that the earliest cellular life on Earth appeared 3.8 billion years ago (Ga) (Betts et al., 2018). Evolution of life in terms of different forms and functions of the primitive cellular life, emerged as organisms were divided into three domains; Bacteria, Archaea, and Eukarya (Madigan et al., 2017) (**Figure 5.1**). Tracing the origin of these three domains with molecular evidence of their gene sequences, it proved that all of them have a common ancestor, the last universal common ancestor (LUCA) and bacteria was the oldest living species among them (**Figure 5.1**). The atmosphere in the early Earth (> 2 Ga) was anoxic and life on Earth exposed to harsh UV radiation, which lead to only anaerobe could survive under these conditions. Following, phototrophic microorganisms emerged on Earth and oxygenic phototrophs, such as cyanobacteria started to produce oxygen into the atmosphere and oceans of Earth. This oxygenation of Earth's atmosphere and oceans supported biological evolution, which boosted the diversity and evolution of prokaryotic microbes (Javaux, 2006, Madigan et al., 2017).

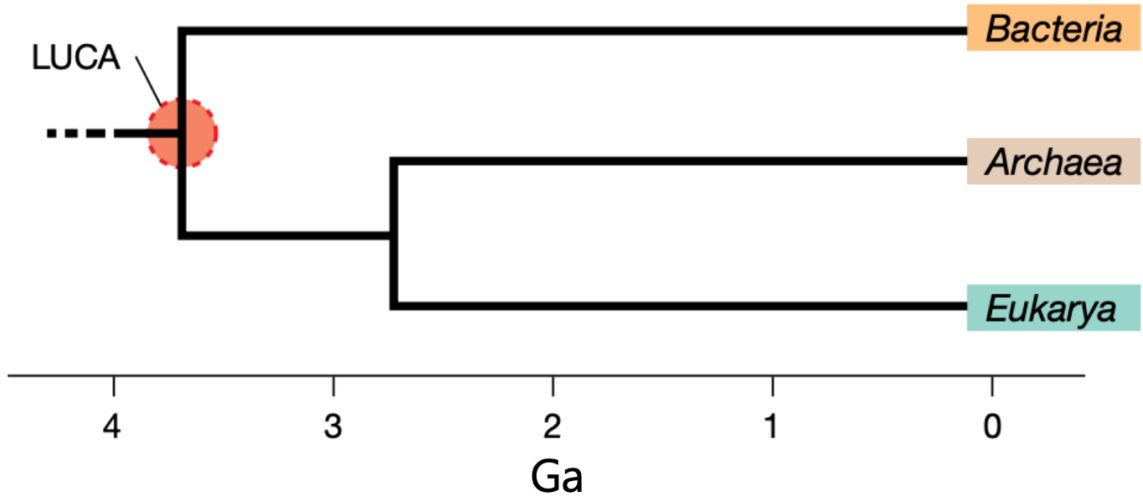


Figure 5.1. The three domains of cellular organisms are Bacteria, Archaea, and Eukarya. LUCA: last universal common ancestor (Madigan et al., 2017).

The evolution of bacteria is different compared to the other two domains in some

respects. First, bacteria are considered as asexual and haploid, although possess a number of mechanisms for horizonal gene transfer, thus allowing the asymmetrical exchange of genetic material can occur skipping reproduction (Madigan et al., 2017). According to the genome analysis, there are two types of genomes in bacteria: the core genome and the pan genome. The number of genes in genomes of the different strains from the same bacteria varies depending on the environment, which shows that bacterial genomes are highly dynamic. The genes in the pan genome contain genes from other microbes through horizontal gene transfers which is considered to promote the evolution (Madigan et al., 2017). As environmental conditions on early Earth were extreme, which may be common for other planets, microbes living in adverse such conditions (also known as extremophiles) are of research value and practical significance (Javaux, 2006).

5.1.2 Microbial signatures beyond Earth

The microbial signatures which can be preserved and expressed vary from their morphological, organic, and metabolic characteristics of the microbes (Westall et al., 2015). In terms of the morphological structures, microbes can be preserved as themselves as well as their products, such as extracellular polymeric substances (EPS); and associations of cells, such as colonies, microbial mats, and bioconstructions (i.e. stromatolites) (Westall et al., 2015). For organic materials, microbes and their products (such as EPS) can be degraded to small molecules remained into inside mineral particles. These molecules can be traced back to their origin or even to identify their domains. For instance, carotenes can be considered originally from carotenoids and, steranes originate from steroids (Summons, 1993). Furthermore, the degraded carbon from microbes in minerals can also be regarded as the biomarkers to trace the origin of life (Summons, 1993, Derenne et al., 2008).

In the quest for identifying microbial life in extraterrestrial settings, metabolic markers play a pivotal role. These markers are essentially biochemical byproducts generated through microbial metabolic processes, with gases and isotopic fractionations of elements such as carbon and sulfur being among the most commonly examined

indicators (Westall et al., 2015). It is crucial for these bio-signatures to be initially incorporated into sedimentary matrices to minimize degradation from environmental factors. Geological events like volcanic eruptions may alter these preserved features over time (Westall et al., 2015). Moreover, it is important to consider the potential influence of exogenous organic molecules. These can be sourced from interplanetary dust particles (IDPs), micrometeorites, and meteorites, and may also be detectable on rock surfaces. For instance, carbonaceous chondrites are known to be composed of approximately 70% insoluble organic matter (IOM), predominantly polyaromatic hydrocarbons (PAHs), and about 30% soluble organic matter (SOM), which includes biologically relevant molecules such as amino acids and carboxylic acids (Pendleton and Allamandola, 2002, Westall et al., 2015, Botta et al., 2002). The spatial distribution of such exogenous organic matter of abiotic origin on Mars' surface is hypothesized to be both extensive and heterogeneous. These compounds can be delivered and disseminated through hydrothermal fluids associated with volcanic or impact events, or may be unearthed through processes like impact gardening (Westall et al., 2015). These complexities underscore the need for a multifaceted approach when searching for signs of microbial life on extraterrestrial bodies.

5.1.3 Contamination issues in the search of life

Terrestrial contamination is an important factor in the study of searching life signals for planetary mission. However, the most common organic biosignatures found in meteorites are considered they are not contaminated by the terrestrial environment. For instance, some unknown amino acid was detected in the carbonaceous chondrites which was not existing on the Earth (Pizzarello, 2007). Extracting soil samples on the surface of Mars is similar to extracting core samples from below the seafloor, and by reviewing this process can be considered as the reference value (Lever et al., 2006). To specific, the potential contamination for extracting the core samples under the seafloor was the seawater running into the borehole. The solution to reduce the contamination was to clean the exteriors of collected samples, and/or eliminate external material from sample or sediment cores (Cavalazzi and Westall, 2019). DNA sequencing methods were used

to estimate the contamination of core material, and chemical tracers and microsphere beads were also used to evaluate the contamination especially for microbiological contamination (Smith et al., 2000). In the research of Friese et al. (2017), it presented a simple and inexpensive way based on the microspheres to assess the degree of contamination, which was using an aqueous fluorescent pigment dispersion. The use of fluorescent particles (the concentration) was quite similar to the microsphere tracers but the cost was much cheaper. Collection and recovery of soil samples from the surface of Mars also faces the problem to contamination from the instruments equipped on rovers and the process to transfer samples to detectors. The contaminants from the external environment and the instruments themselves may influence the result of the microbiological and many other analyses. Therefore, contaminants cannot be ignored as it is of crucial importance to develop methods to evaluate the degree of contamination and detect uncontaminated soil samples.

5.1.4 The search for signs of microbial life

It is hard to identify living microorganisms on the surface of Mars for various reasons. Not only could radiation from the sun, galaxy and universe degrade volatile organic molecules, but oxidants which exist on Martian soil, such as perchlorates, could oxidize any organic molecules once they reach a certain temperature (Atreya et al., 2006, Summons et al., 2011). Many rovers used for Mars mission, such as Curiosity, ExoMars, and even on Mars 2020, have instruments equipped for *in situ* experiments of samples on Mars. However, due to the existence of perchlorates, which can be activated and destroy organic molecules during the analysis process, there are some difficulties in detecting and identifying of organic molecules on samples. In addition, the detection efficiency can vary among the different techniques which depend on the extraction and delivery of the samples to the detector (Glavin et al., 2013).

In 1972, the Viking mission launched three life-detection metabolic tests on the surface of Mars (Levin, 1972): the Labeled Release Experiment (LR experiment), the Gas Exchange (GEx) experiment and the Pyrolytic Release (PR) experiment. The design of LR experiment encompassed the detection of carbon-based gases hypothesized to result

from microbial metabolic activity, and it delivered a positive result. The GEx experiment delved into the diverse conditions that could potentially influence microbial life, including dry, wet and a sophisticated concoction of organic nutrients and supplements referred to as “chicken soup”. Meanwhile, the PR experiment focused on the observation of microorganismal photosynthesis through the hunt for demonstrable carbon fixation evidence (Levin and Straat, 2016). Although the Viking mission ultimately posited the detection of life on Mars, this assertion emerged as a profoundly controversial conclusion. Notably, in the years following the Viking mission, the Phoenix lander's discovery of perchlorate, a potent oxidizing agent, raised skepticism regarding the conclusions drawn from the LR experiment. This is due to the capacity of perchlorate to degrade organic compounds and discharge carbon dioxide through strictly chemical means, thus providing a nonbiological explanation for the LR experiment's results (Wadsworth and Cockell, 2017).

While the nonbiological interpretation of the Viking LR results provides an intriguing perspective, several unresolved issues remain. There is no established evidence of perchlorate or its radiation by-products at the Viking LR experimental sites (Levin and Straat, 2016). Moreover, the LR agent on Mars is subject to alteration when exposed to temperatures around 50°C and can be negated by prolonged storage in darkness at 10°C, conditions that are conducive to biological activity (Levin and Straat, 2016). Thus, despite the captivating abiotic interpretations of the LR data, these cannot be considered as irrefutable evidence, which leaving the door open for biological explanations.

Historically, studies such as the Viking biology experiments have focused on the incubation of soil microorganisms as a means to identify metabolic evidence. However, a comprehensive method for detecting microbial growth in Martian soil still needs to be discovered. Within a controlled laboratory environment, vegetative polyploid microorganisms showcased an appreciable enhancement in radiation resistance following five days of desiccation and exposure to -80°C. Simultaneously, research on endolithic bacteria underscored the potential for microbial survival within rock pores and fissures, even under harsh environmental conditions (Horne et al., 2022),

reinforcing the resilience of microbial life. Therefore, Martian environmental conditions could potentially facilitate the existence of dormant microorganisms or extremophilic bacteria concealed beneath the planet's surface (Yamagishi et al., 2014, Kounaves, 2007), thus widening the scope of astrobiological research.

5.1.5 Challenges and limitations in detecting microbes on Mars

The quest to detect microbial life on Mars is confronted with various challenges and limitations. Understanding and addressing these factors are crucial for developing reliable methodologies and instruments for the accurate identification and characterization of potential microbial life on the Martian surface. The biggest challenge is the formidable environment on Mars, characterized by harsh conditions such as low temperatures, high radiation levels, thin atmosphere, and scarce liquid water availability (Cockell et al., 2000). These conditions can significantly impact the survival and metabolic activities of potential microbes, making it challenging to detect and study organisms that have adapted to such harsh conditions.

Further challenges in detecting Martian microbial life arise from the anticipated low biomass and minimal metabolic activities of possible life forms (Wilhelm et al., 2018). The extreme conditions and limited available resources may result in slow-growing microbial populations with restrained energy consumption and limited production of detectable metabolic byproducts (Gupta et al., 2017). Therefore, the design and implementation of highly sensitive detection methodologies capable of discerning subtle indications of life pose a considerable challenge.

Moreover, the existence of indigenous Martian compounds further complicates microbial detection. The identification of perchlorates instigates worries about potential chemical reactions with organic compounds (Levin and Straat, 2016). These reactions can lead to the release of carbon dioxide in purely chemical processes, thereby yielding false-positive or false-negative results in life-detection experiments. Consequently, the interpretation of experimental data requires careful consideration of the role and effects of these indigenous compounds.

The procurement of representative Martian samples without introducing contamination during collection and handling processes is another formidable challenge. Adherence to planetary protection guidelines, stringent protocols, and sterile techniques is imperative to maintain sample integrity and avoid false positives or unintentional introduction of terrestrial microorganisms (Swindle et al., 2022).

The limitations inherent to the equipment used for microbial detection on Mars present additional hurdles. Life-detection instruments vary in sensitivity, specificity, and detection thresholds, requiring cautious calibration and validation. The inherent complexity of biological systems and the ambiguous signatures of microbial activity mandate the utilization of auxiliary techniques and multiple detection methodologies for reliable and precise outcomes (Zhang et al., 2021). Careful interpretation of data, distinguishing biological from abiotic processes, and the integration of contextual information are vital for robust conclusions (Bapat and Rajamani, 2023).

Nevertheless, technological advancements, including miniaturized tools, biosensors, and molecular biology techniques, offer promising strategies to surmount these challenges (Naresh and Lee, 2021, Poghosyan and Golkar, 2017). These innovations hold the potential to enhance the sensitivity, specificity, and interpretative accuracy of data, contributing to a more definitive evaluation of potential microbial life on Mars.

By acknowledging and addressing these impediments and limitations, the scientific community can promote the evolution of novel methodologies and tools for Martian microbial life detection and characterization. This progress will yield invaluable insights into Mars's habitability and the potential for extraterrestrial life forms.

5.1.6 Structure of the chapter

This chapter focuses on the optimization of simplistic methodologies utilizing a range of culture media (broth and agar media) to directly detect microbes from soil samples. The primary objective of this approach is to enable the visual identification of life signs, thereby circumventing interference from physicochemical reactions. This chapter outlines the experimental procedures and documents the ensuing results obtained from

the optimized approach in a dedicated section. A comprehensive description of the visual signs of life detected and a discussion on their implications are provided. In addition, this chapter also describes the examination of the impact of ultraviolet (UV) radiation on microbial life within a simulated Martian milieu, given that UV radiation is a prominent stressor that Martian microorganisms would confront due to the absence of a substantial atmosphere and the lack of a protective ozone shield. The chapter finishes with a summary encapsulating the primary findings, an interpretive discussion on their implications, and suggested trajectories for future research. This overarching goal of this chapter is to provide a comprehensive insight into the process of optimizing direct microbial detection methodologies, thus expanding our understanding of microbial life.

5.2 Materials and methods

5.2.1 Bacteria culturing and media preparation

Luria Bertani (LB) broth media was prepared by dissolving 20 g of LB broth powder in 1 litre of ddH_2O. The broth was transferred to Duran bottles and sterilised by autoclaving for 15 minutes at 121°C. Sterile LB broth media was stored at 4°C. For soli media LB agar and nutrient agar were used. LB agar media was prepared by dissolving 32 g of LB agar powder in 1 litre of ddH_2O. Nutrient agar media was prepared by dissolving 28 g of nutrient agar powder in 1 litre of ddH_2O. Media was transferred to Duran bottles and sterilised by autoclaving for 15 minutes at 121°C. Sterile LB or nutrient agar media was transferred ml in sterile Petri dishes and stored at 4°C. Phosphate buffer saline (PBS, pH 7.4) was prepared as 10x concentrate and diluted with ddH_2O to 1x and sterilised and stored at 4°C after autoclaving.

5.2.2 *Escherichia coli* culturing and soil spiking

Escherichia coli (E. coli) (the strain is from NCIMB - catalogue number 9485) was cultured at 37°C under agitation at 150 rpm in LB broth media. From a liquid *E. coli* culture in LB broth media, a bacterial suspension was prepared in Eppendorf tubes and centrifuged (to clear media) at 900 *g* for 4 minutes at room temperature. The supernatant was removed, and the pelleted bacteria were re-suspended in PBS. Bacteria were precipitated once again by centrifugation at 900 *g* for 4 minutes and the supernatant was discarded.

For spiking of live bacterial cells into the soil, the bacterial pellet was re-suspended in sterile PBS. The absorbance of the suspension was quantified at 600 nm, with an absorbance of 1 unit at this wavelength denoting approximately 800 million cells per ml based on the McFarland standards (McFarland, 1907). A suspension with an absorbance of 0.1 (representing approximately 80 million cells/ml) was prepared and diluted as required, then spiked into the soil samples.

Soil simulants (JSC Mars-1A Martian Regolith Simulant) was sterilised by autoclaving

for 15 minutes at 121°C in glass vials. In a sterile glass vial with 100 mg sterile soil, 10 µl with different concentrations of *E. coli* solutions in PBS was added and allowed to dry on the bench with the cap tightly closed to let it dry. It was suspected that the bacteria would remain in the soil and, therefore, subsequently either 2 ml LB sterile broth would be added directly into vials to incubate at 37°C or 2 ml PBS to extract the bacteria which would then be transferred to Petri dishes with solid media to incubate at 37°C. The bacteria in broth media were quantified by measuring the absorbance at 600 nm, while in Petri solid media colonies were counted (**Figure 5.2**). To ensure the accuracy and sterility of the conditions, control groups were prepared using non-spiked soil as well as empty sterile vials without soil. The overall spiking process was optimized and will be described in the Results section.

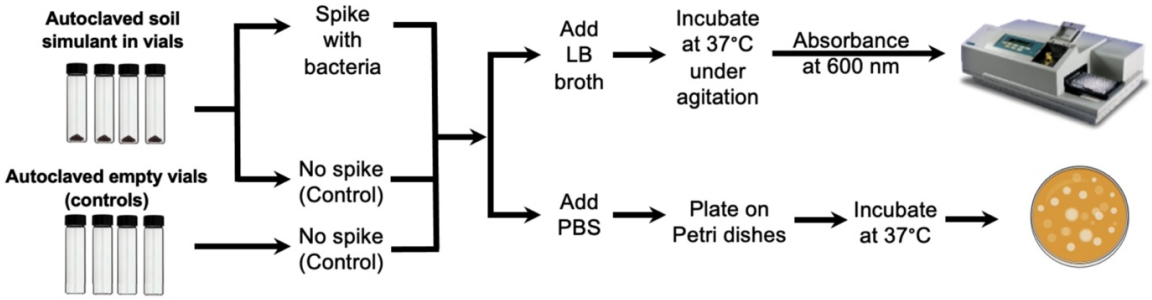


Figure 5.2. Spiking, recovery and detection methods of soil samples with *E.coli.*

5.3 Results

To troubleshoot the approach which was described in the previous section for the detection of bacteria, initially LB broth was explored in relation to the different concentration of bacteria which were spiked on JSC. Bacteria were re-suspended in sterile PBS and diluted from 10^3 to 10^9 cells/ml and then spiked on sterile JSC in glass vials. After drying at room temperature, LB broth media was added directly into the vials and incubated at 37°C under agitation at 150 rpm in an incubator. In order to eliminate any potential contamination sources, controls comprising of medium with no soil added and medium with non-spiked soil were concurrently processed. After 24 hours incubation, the media were separated from the soil, and the absorbance was measured at 600 nm to estimate bacterial growth (**Figure 5.3**). This finding indicated that bacteria spiked on the JSC could be successfully detected via the LB broth media, with a detection threshold as low as 20 cells per 50 mg of soil. Notably, a plateau was observed upon spiking of 2 x 10^7 cells on 50 mg of soil.

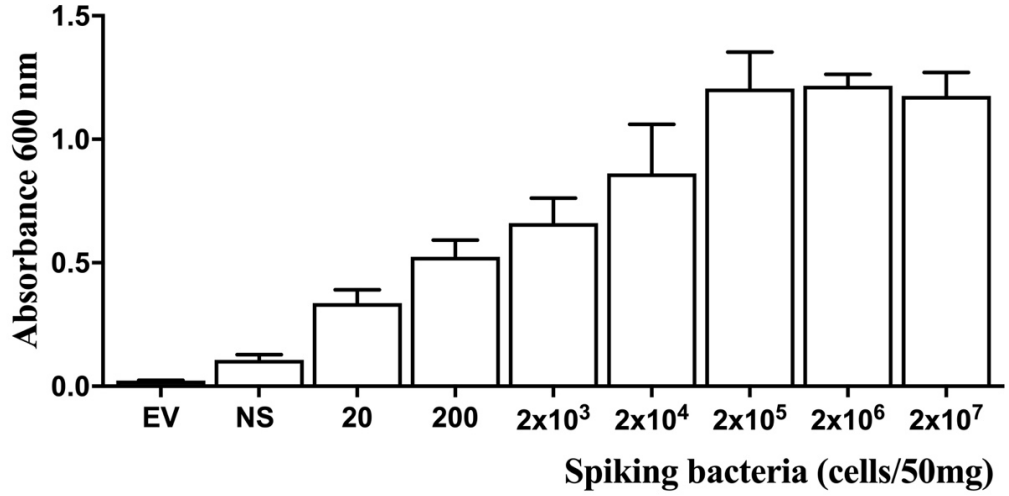


Figure 5.3. Spiked *E. coli* in soil detected by the LB broth media. Data represent average±SD (N=4). EV: Empty vial control of LB broth without cells or soil, NS: non-spiked soil sample.

The stability of LB broth following a period of freezing was also assessed as a viable preservation technique for the media during potential interplanetary voyages to Mars, which would necessitate a storage phase prior to utilizing the media for experimental

tests. Both freshly prepared LB broth media and LB broth media frozen and stored at -20°C for three months were used to detect bacteria spiked on the soil, facilitating a comparative analysis (**Figure 5.4**). The protocol adhered to for this experiment mirrored that of the previously detailed method. The results indicated a negligible difference between the fresh and frozen LB broth media. Both forms of media demonstrated their efficacy in detecting the spiked bacteria, thereby validating the feasibility of long-term frozen storage of LB broth media without risk of contamination. This finding serves to emphasize the potential utility of media in future experiments aimed at bacterial detection, and the capacity to foster microbial growth.

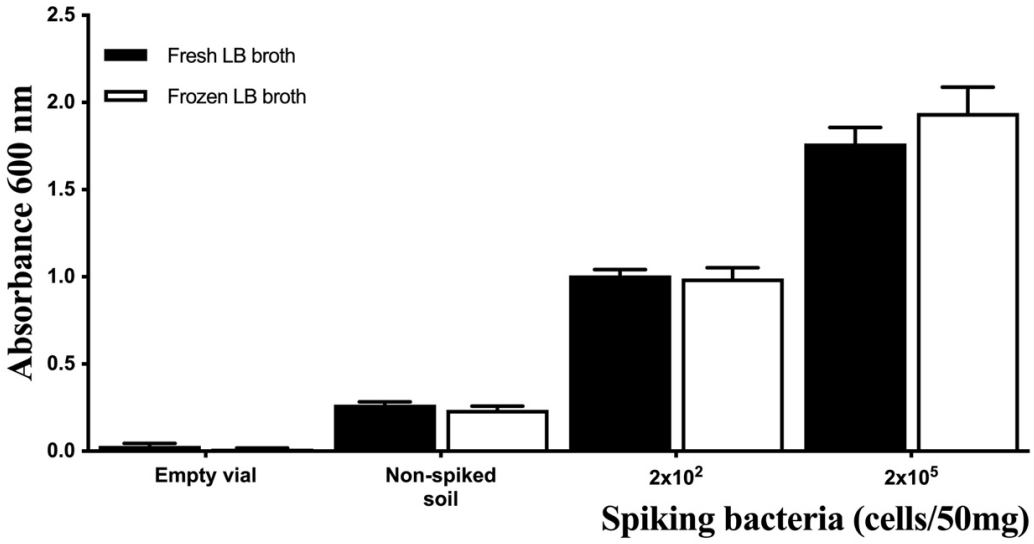


Figure 5.4. Comparison with frozen and fresh LB broth media to detect spiked *E. coli* in soil. Data represent average±SD (N=4).

Following, the impact of the presence of soil (JSC simulant or soil from campus (CS)) was assessed in suspension (**Figure 5.5A**) or after spiking and recovery from the soil (**Figure 5.5B**). The results showed that the presence of both soils will not inhibit the growth of the bacteria either in suspension or spiking.

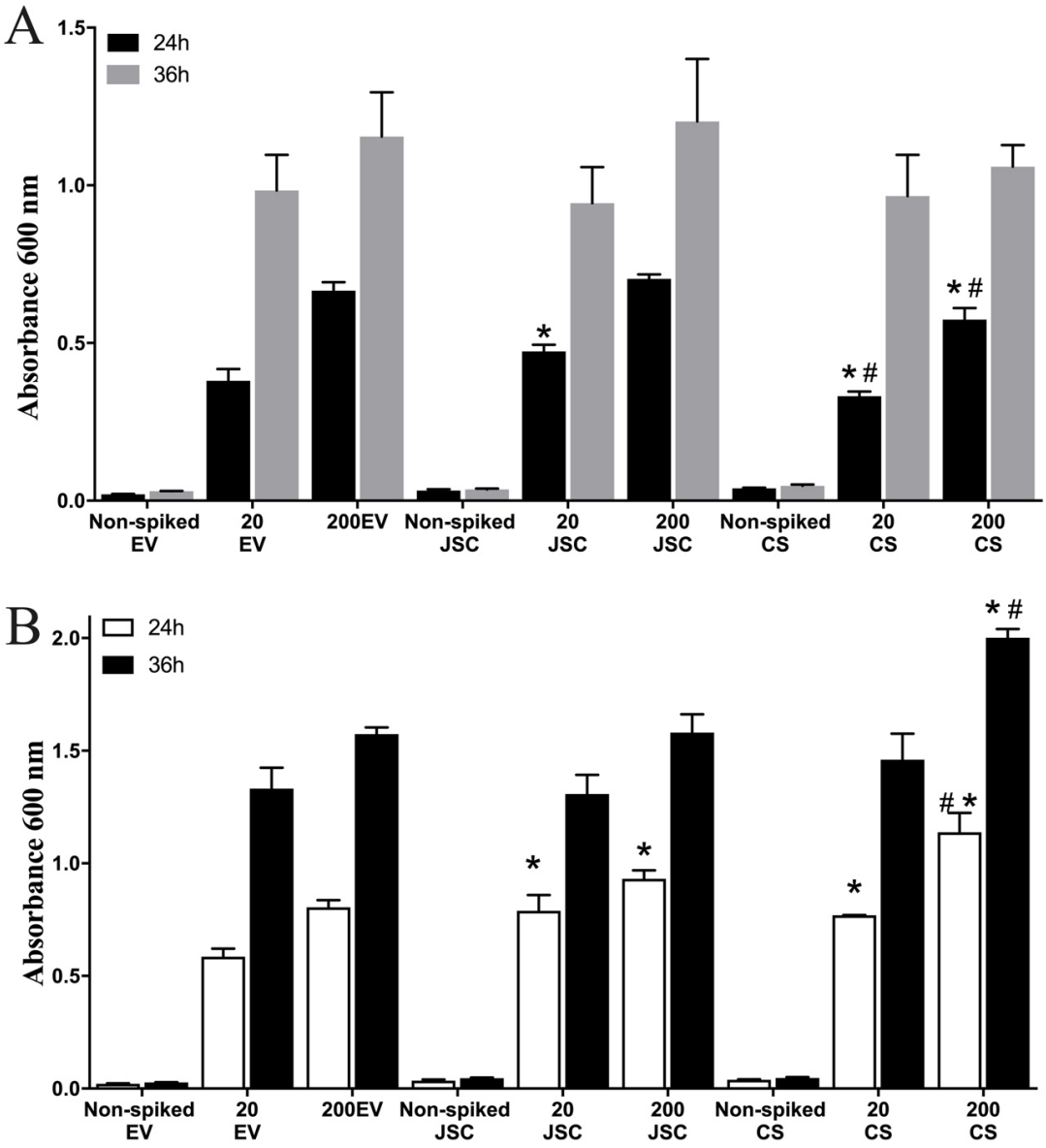


Figure 5.5. The impact of soil (JSC simulant or CS from campus) was assessed in suspension (A) or after spiking and recovery from the soil (B). EV: Empty vial control of LB broth without cells or soil, NS: non-spiked soil sample. Data represent average±SD (N=4). Statistically significant changes were assessed with a two-way ANOVA (or mixed model) statistical analysis in GraphPad compared with the EV (*) or JSC (#).

It is expected that UV radiation on the Martian surface would pose detrimental conditions to microbial survivability. Based on the study of Diaz and Schulze-Makuch (2006), JSC spiked with bacteria were subjected to UV exposure at 37 W/m^2 by using

CL-1000 Ultraviolet Crosslinker from UVP (**Figure 5.6**). Bacteria were a) extracted from soil samples in sterile PBS or added directly to LB broth; or b) added directly to the LB agar media, in order to present alternative methods for microbial life detection. Bacteria were incubated at 37°C for 24 hours and allowed to grow. The growth assessment for the bacterial culture was carried out differently for broth and agar media. For the former, the absorbance was determined, while the latter was evaluated through imaging of the colonies. This dual approach afforded a comprehensive analysis of bacterial growth under UV-exposed conditions.

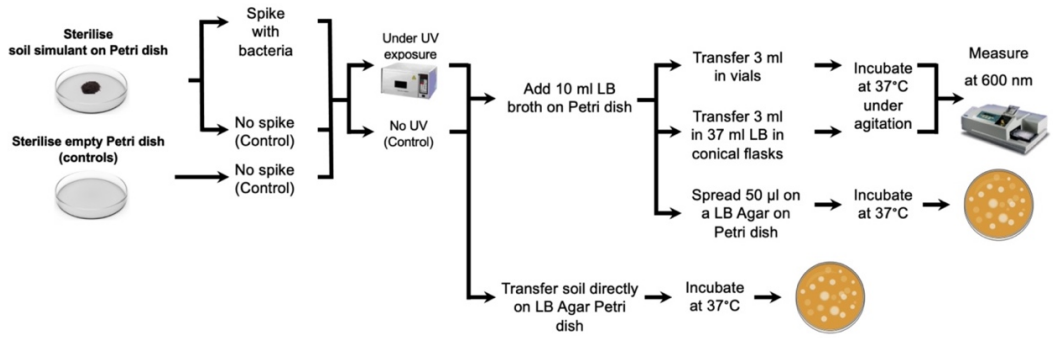


Figure 5.6. Method of spiking bacteria for UV exposure.

In order to determine the effect of UV radiation on bacterial populations, samples of JSC spiked with bacteria and their respective controls were examined. Initially, the impact of UV exposure on bacterial growth was evaluated in a time-dependent manner using LB broth as the growth media (**Figure 5.7**). Spiked JSC was exposed to UV radiation for various durations, extending up to 240 minutes. and following allowed to grow after extraction in LB broth in conical flasks. The clarity of all control samples indicated an absence of contamination, thus ensuring the validity of the experimental results. These observations conclusively demonstrated that UV radiation can indeed impose inhibitory effects on bacterial growth.

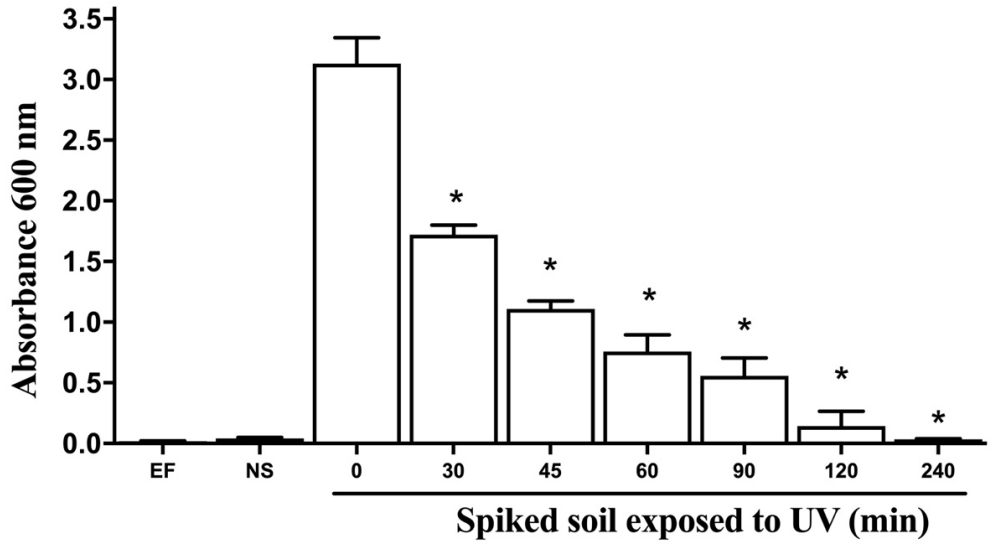


Figure 5.7. The impact of UV radiation to bacteria spiked on soil, bacteria grew in LB broth. NS: non-spiked soil sample. Data represent average±SD (N=4). EF: Empty flask control of LB broth without cells or soil, NS: non-spiked soil sample. *Statistically significant changes were assessed with a two-way ANOVA (or mixed model) statistical analysis in GraphPad.

For the purpose of simplifying the detection approach and to enable more straightforward visual recognition of bacterial life extracted from the soil matrix, the utilization of agar culture media was explored in this research. *E.coli* were initially spiked on the JSC and subsequently exposed to UV radiation. Following this, the spiked-JSC samples were permitted to cultivate directly on LB Agar media, bypassing any form of extraction, or alternatively after extraction in PBS.

Different concentration of *E.coli* was spiked on JSC and then exposed to UV radiation for up to 60 minutes before being cultured on LB agar (**Figure 5.8**). All controls were clear without any microbial growth, which indicated that there was no contamination issue from the experiment, and that the observed colonies originated solely from the bacteria spiked soil samples. The observation of bacterial detection using LB agar, and the associated decrease in bacterial colonies, corroborated the hypothesis that UV radiation could significantly hamper bacterial survival, thereby simulating the hostile conditions present on the Martian surface.

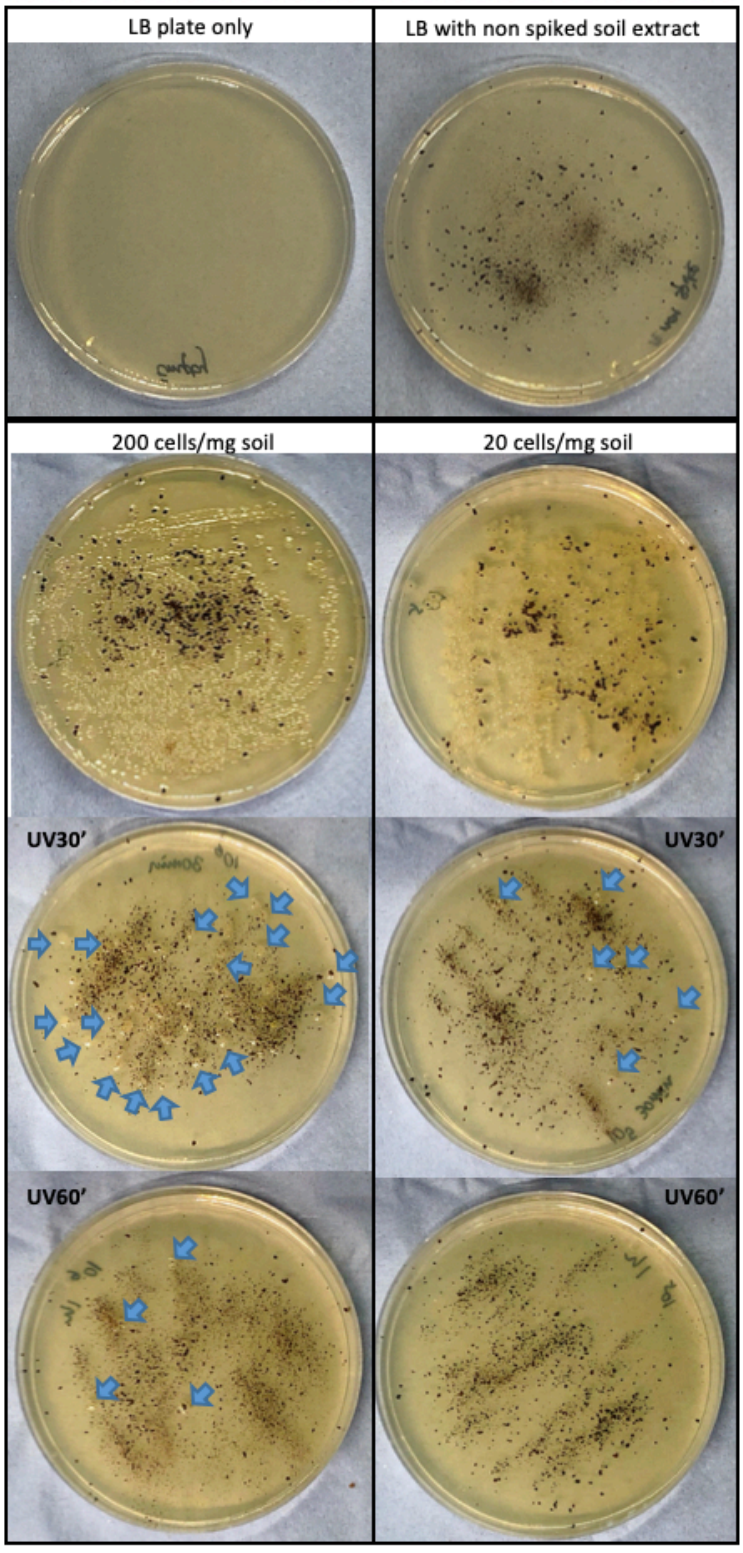


Figure 5.8. The impact of UV radiation to bacteria spiked on soil. Bacteria were cultured on LB agar directly from soil samples. Figures represent indicative plates from quadruplicates. Arrows show colonies in the UV treated plates.

To validate the efficiency of the spiking process and corroborate that all observed colonies originated from the spiked soil, soil samples were spiked with bacteria at different concentrations (**Figure 5.9**). The absence of microbial growth in the control samples and the increasing colony counts that corresponded with higher spiking concentrations reinforced the absence of contamination issues and the successful introduction of bacteria into the soil samples.

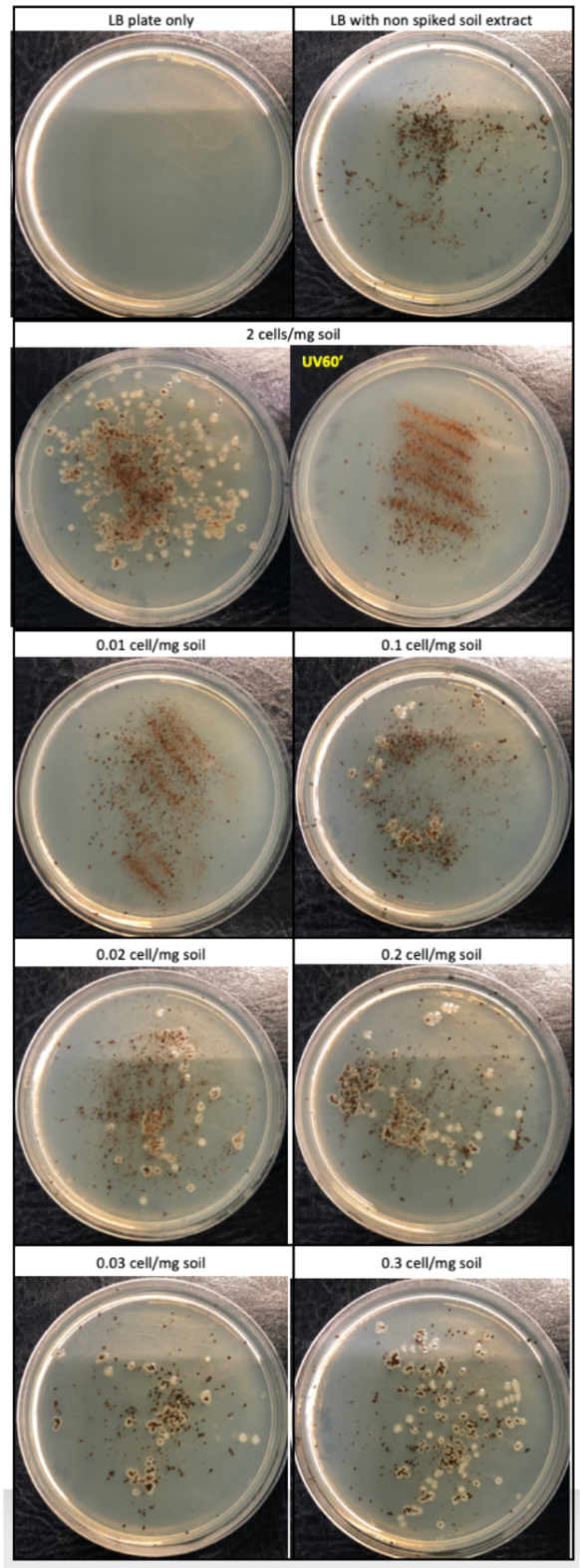


Figure 5.9. Spiking of different number of bacteria on soil. Bacteria were cultured on LB agar directly from soil samples. Figures represent indicative plates from quadruplicates.

Following, an alternative method was instituted, whereby the spiked soil was extracted in PBS before being spread across an LB agar plate (**Figure 5.10**). This method was chosen for its ability to yield clearly demarcated colonies on the agar plate. As anticipated, UV radiation was once again proven to impede bacterial growth within the soil, further validating the initial experimental findings.

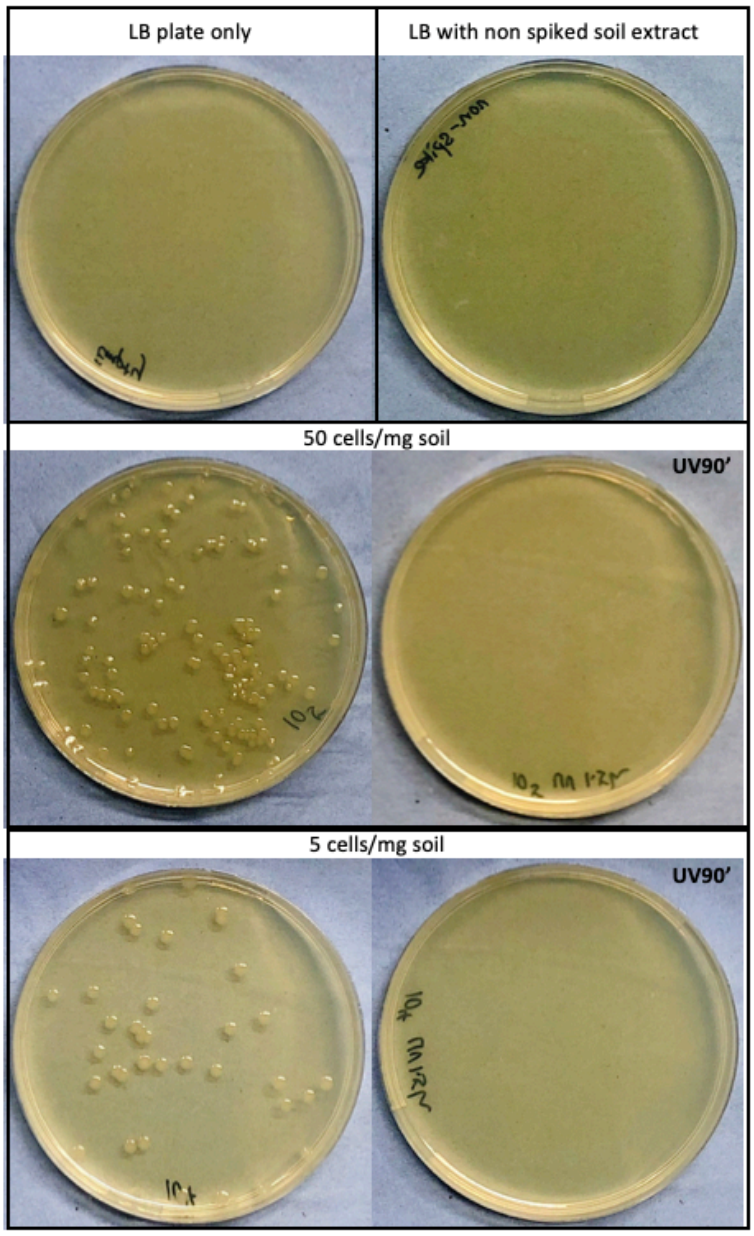


Figure 5.10. The alternative approach of extracting spiked bacteria from soil in PBS and culturing on LB Agar. Bacteria were extracted, spread, and cultured on LB agar to grow colonies. Figures represent indicative plates from quadruplicates.

5.4 Discussion

In order to detect the life activity, specifically the growth of life for the planetary missions, this research introduced two types of media: broth and agar media. The experimental design involved simulating Martian conditions by spiking the JSC soil matrix with *E.coli* and subsequently exposing the samples to UV radiation. The spiked soil samples were then either extracted in PBS for growth in the media or directly incorporated into the media for microbial cultivation. Evaluation of the non-spiked JSC soil matrix indicated an absence of microbes, or at the very least, a microbial count that fell below the detection threshold. Upon incrementally increasing the concentration of bacteria, it was demonstrated that both the LB broth and agar media could be successfully employed for the visual detection of microorganisms.

This approach is much simpler and straight-forward compared with the polymerase chain reaction (PCR) method (Picard et al., 1992) and other sequencing approaches. In the study of Picard et al. (1992), a rapid microbes detection assay was proposed based on PCR technology. Due to the existence of humic acids and phenolic compounds in soil on Earth, which can contaminate the DNA extracted and affect the accuracy of the final PCR result, Picard optimised all the cricial steps including direct lysis of cells (sonication, microwave heating, and thermal shocks), DNA purification (using Elutip d columns), and PCR amplification (using booster conditions, lower denaturation temperatures, and the addition of formamide). However, it's acknowledged that the composition of Martian soil differs significantly. Martian regolith is devoid of organic compounds, including humic acids and phenolic compounds, presenting a distinct set of challenges for microbial growth but an opportunity for microbial detection. Undoubtelly, this PCR-based method can detect microbes in soil rapidly and specifically, however, it required several essential equipments to apply this test, such as columns to purify DNA and the PCR machines. Therefore, as an approach it cannot be easily applied to the planetary missions for the *in situ* microbes detection. Another method for the detection of microbes has been based on the biological redox signature of microbes (Crawford et al., 2002). The electron transport chain is a prominent feature

in living organisms to obtain energy. Therefore, using respiratory electron-accepting dyes to analyse these specific electron transport molecules, such as porphyrins and/or quinones can prove the existance of life in soil. The respiratory dyes include 2,3-dichlorophenol indophenol (DCIP) and the tetrazolium dye 2,3-bis(2-methoxy-4-nitro-5-sulfophenyl)-2H-tetrazolium-5-carboxanilide inner salt (XTT), which can participate in the microbial electron transport and be reduced, this phenomenon can be captured by visible spectroscopy. This method can be use to the detection of microbial activity in soil *in situ*, however, it also required special equipments for the extraction of soil and the separation of extracted compounds, which could be a challenge to operate in space.

The exploratory processes undertaken in this study validated the overall feasibility of the developed methodologies for identifying microbial life in the context of planetary exploration and confirming microbial proliferation from soil matrices. In essence, the proposed approaches proved to be highly reproducible, thereby ensuring the sterility of stored media and efficiency, particularly in scenarios where *in situ* preparation may not be viable. These advancements pave the way for novel exploratory tools and offer alternative strategies for microbial life detection, propelling research in this field. However, it's critical to consider the potential limitations and challenges associated with the interpretation of the results gleaned from this proposed method.

One of the foremost challenges is the high levels of radiation present on the Martian surface. Using UV radiation exposure as the simulation of these harsh conditions confirmed the detrimental effects of such radiation on microbial recovery. However, subterranean Martian conditions may provide a habitable environment (Jones et al., 2011). Long-term ionizing radiation exposure of Martian soil microorganisms suggests their best-case scenario for long-term survivability (exceeding one million years) lies beneath depths of 2 meters (Dartnell et al., 2007). This suggests microorganisms in the upper 2 meters are unlikely to survive beyond a few million years.

However, research involving radiation-exposed microorganisms indicates certain species possess radiation-resistant characteristics. Musilova et al. (2015) demonstrated the irradiation and desiccation survival traits of Halomonads. Interestingly, this study

suggested that irradiation at -80°C (typical temperature of the Martian surface) could enhance microbial radiation resistance. The Mars EXposed Extremophiles Mixture (MEXEM) program reported up to three months of survivability for facultative anaerobic microbes, such as Salinisphaera shabanensis and Buttiauxella sp., under anoxic conditions with extreme radiation and desiccation (Beblo-Vranesevic et al., 2022). Additionally, the orbital-driven oscillations of Martian climate may occasionally allow for the transient availability of liquid water, potentially enabling microorganisms to repair damage, propagate, and revert to dormancy (Laskar et al., 2004). These findings suggested the potential for microbial survival on Mars, lending credence to the practicality of our growth media, especially when considering deeper samples to access viable microorganisms.

In extreme environments, bacteria have been found to enter a viable but non-growing state, necessitating specific stimuli to initiate growth (Puspita et al., 2012). While the ultimate objective of this project does not involve cultivating diverse microorganisms under varied conditions, it is aim to establish a direct and rapid method for detecting microbial life in Martian soil analogues. It is acknowledged that using selective media may introduce challenges; however, the approach proposed in this study can be tailored to target specific microbial species, taking into account their unique growth requirements. This study is intended to serve as a foundation for future research in this domain, aiding the development of more sophisticated methods for identifying and cultivating extraterrestrial microorganisms.

Another concern for the long-term survivability of microorganisms on the surface of Mars is the presence of reactive oxidant species in Martian soils, such as peroxides and reactive chlorine species generated from radiolytic perchlorate decomposition (Quinn et al., 2011). Wetting Martian soils with an organic solution triggers reactive chemistry similar to the processes observed by the Viking landers during the labelled release experiment, leading to rapid organic oxidation by H_2O_2 and reactive chlorine species. In separate research, it was shown that perchlorate presence amplifies the biocidal effects of UV light (Wadsworth and Cockell, 2017). These findings suggested that the

survivability of Martian soil microorganism may be lower than earlier estimates. Consequently, while this study focuses on the development of a method to detect potential Martian microorganisms, the potential influence of perchlorates and other reactive oxidant species is acknowledged. Future research should continue to investigate the role of these oxidants in Martian soils and their implications for life detection on Mars.

Furthermore, from a recent report, there were three methylobacterium species discovered on the international space station (EurekAlert and AAAS, 2021). Due to the features of methylobacterium species which are associated with phosphate solubilization, nitrogen fixation, abiotic stress tolerance as well as plant growth promotion and biocontrol activity against plant pathogens, this discovery provided the hypothesis and posibility to grow plants outside of Earth and promote the development of space farming. The report also stated that there were approximately 1,000 samples were waiting for returing to Earth to analyse. Hence, developling a rapid and simple method to the initial tests for microbes in space is necessary for achieving a sustainable space exploration. The assay presented in this chapter was based on the liquid and solid media which can achieve this goal for the initial detection of bacteria. However, there are still some potential factors which could affect the accuracy of the assay such as the sterile environment required all the time, which is crucial to ensure the experiment and that there is no contamination from external environment, including the handling equipment. In conclusion, with this chapter, an optimized method for the detection of microbial life on soil simulants was developed as an easier alternative way to detect bacteria from the soil matrix. The necessary steps of spiking, extraction and confirmation were optimised for the feasibility of such approach to develop into a novel planetary exploration tool and present alternatives for the detection of microbial life that will drive forward this research field. No matter if the focus of this work is planetary exploration, the approach presented here could be applied to Earth soil such as in the context of quantifying the microbial content of soil i.e. for assessing the presence of microbes from pollution r suitability for culturing.

For potential translation of these findings to future platforms, miniature agar media adaptations could be deployed in multi-well plates or incorporated into microfluidic methods such as the BactoBox (SBT Instruments, Herlev, Denmark) (Hartmann et al., 2021). Such tactics could augment existing sequencing tests (Goordial et al., 2017) designed for detecting microbes in soil samples within planetary science. These findings dovetail with recent studies on microbial exposure to simulated stratospheric conditions (Cortesao et al., 2021), potentially advancing our understanding of microbial survival under harsh conditions.

Existing space mission scientific instruments largely target the identification of habitable environments or biosignatures. These instruments, often large rovers, entail substantial costs and mission failure risks (Vago et al., 2017). Despite their ability to analyse specific molecules or organics via designated compartments, an alternative strategy might be the development of smaller, independent platforms suitable for low-cost, specific missions. Miniaturised culturing approaches could prove invaluable in smaller mission instruments for sampling soil, testing for microbial life, or assessing inhibitory impacts on microbial growth.

5.5 Conclusions

In conclusion, the chapter has delivered a comprehensive investigation into the challenges and potential approaches for recognising microbial life within simulated Martian soil. The utilisation of two forms of media, specifically, broth and agar LB, has been scrutinised for their effectiveness as direct, rapid techniques for identifying microbial existence. The ability to implement these methodologies, even in the severe conditions present on Mars, confirms a crucial advancement for the feasibility of life detection in future planetary missions. The approach has demonstrated high specificity and sensitivity, as shown by the successful detection of *E. coli* in soil simulants.

It is vital to recognise that the methodologies developed in this chapter provide a foundation for subsequent research. These techniques, while elementary, have been designed with adaptability in mind - to be tailored to distinct microbial species and their unique growth requirements, thereby offering a flexible tool for future investigations. Furthermore, the research emphasises the need for continued exploration of Martian soils, especially in considering the impact of oxidants on microbial survival.

The future of this field could also consider incorporating more sophisticated techniques, such as miniaturised culture methods and the potential application of microfluidic technologies. Moreover, sequencing tests could supplement these methods to provide a comprehensive strategy for identifying and characterising microbial life in soil samples from Mars or other extra-terrestrial bodies.

While this approach offers a novel starting point for detecting microbial life, a thorough comprehension of the implications of the Martian environment is necessary. This understanding, combined with the continued refinement of detection methods, will help to navigate the challenges and broaden our knowledge in the pursuit of extra-terrestrial life.

5.6 References

ATREYA, S. K., WONG, A. S., RENNO, N. O., FARRELL, W. M., DELORY, G. T., SENTMAN, D. D., CUMMER, S. A., MARSHALL, J. R., RAFKIN, S. C. & CATLING, D. C. 2006. Oxidant enhancement in martian dust devils and storms: implications for life and habitability. *Astrobiology,* 6**,** 439-50.

BAPAT, N. V. & RAJAMANI, S. 2023. Distinguishing Biotic vs. Abiotic Origins of 'Bio'signatures: Clues from Messy Prebiotic Chemistry for Detection of Life in the Universe. *Life (Basel),* 13.

BEBLO-VRANESEVIC, K., PIEPJOHN, J., ANTUNES, A. & RETTBERG, P. 2022. Surviving Mars: new insights into the persistence of facultative anaerobic microbes from analogue sites. *International Journal of Astrobiology,* 21**,** 110-127.

BETTS, H. C., PUTTICK, M. N., CLARK, J. W., WILLIAMS, T. A., DONOGHUE, P. C. J. & PISANI, D. 2018. Integrated genomic and fossil evidence illuminates life's early evolution and eukaryote origin. *Nat Ecol Evol,* 2**,** 1556-1562.

BOTTA, O., GLAVIN, D. P., KMINEK, G. & BADA, J. L. 2002. Relative amino acid concentrations as a signature for parent body processes of carbonaceous chondrites. *Orig Life Evol Biosph,* 32**,** 143-63.

CAVALAZZI, B. & WESTALL, F. 2019. *Biosignatures for Astrobiology*, Springer, Cham.

COCKELL, C. S., CATLING, D. C., DAVIS, W. L., SNOOK, K., KEPNER, R. L., LEE, P. & MCKAY, C. P. 2000. The ultraviolet environment of Mars: biological implications past, present, and future. *Icarus,* 146**,** 343-59.

CORTESAO, M., SIEMS, K., KOCH, S., BEBLO-VRANESEVIC, K., RABBOW, E., BERGER, T., LANE, M., JAMES, L., JOHNSON, P., WATERS, S. M., VERMA, S. D., SMITH, D. J. & MOELLER, R. 2021. MARSBOx: Fungal and Bacterial Endurance From a Balloon-Flown Analog Mission in the Stratosphere. *Front Microbiol,* 12**,** 601713.

CRAWFORD, R., PASZCZYNSKI, A., LANG, Q., ERWIN, D., ALLENBACH, L.,

CORTI, G., ANDERSON, T., CHENG, I., WAI, C., BARNES, B., WELLS, R., ASSEFI, T. & MOJARRADI, M. 2002. Measurement of microbial activity in soil by colorimetric observation of in situ dye reduction: an approach to detection of extraterrestrial life. *BMC Microbiol,* 2**,** 22.

DARTNELL, L. R., DESORGHER, L., WARD, J. M. & COATES, A. J. 2007. Modelling the surface and subsurface Martian radiation environment: Implications for astrobiology. *Geophysical Research Letters,* 34.

DERENNE, S., ROBERT, F., SKRZYPCZAK-BONDUELLE, A., GOURIER, D., BINET, L. & ROUZAUD, J.-N. 2008. Molecular evidence for life in the 3.5 billion year old Warrawoona chert. *Earth and Planetary Science Letters,* 272**,** 476-480.

DIAZ, B. & SCHULZE-MAKUCH, D. 2006. Microbial survival rates of Escherichia coli and Deinococcus radiodurans under low temperature, low pressure, and UV-Irradiation conditions, and their relevance to possible Martian life. *Astrobiology,* 6**,** 332-47.

EUREKALERT & AAAS. 2021. *Three bacterial strains discovered on space station may help grow plants on Mars* [Online]. Available: https://www.eurekalert.org/pub_releases/2021-03/f-3bs031521.php [Accessed June 30 2021].

FRIESE, A., KALLMEYER, J., AXEL KITTE, J., MONTAÑO MARTÍNEZ, I., BIJAKSANA, S. & WAGNER, D. 2017. A simple and inexpensive technique for assessing contamination during drilling operations. *Limnology and Oceanography: Methods,* 15**,** 200-211.

GLAVIN, D. P., FREISSINET, C., MILLER, K. E., EIGENBRODE, J. L., BRUNNER, A. E., BUCH, A., SUTTER, B., ARCHER, P. D., ATREYA, S. K., BRINCKERHOFF, W. B., CABANE, M., COLL, P., CONRAD, P. G., COSCIA, D., DWORKIN, J. P., FRANZ, H. B., GROTZINGER, J. P., LESHIN, L. A., MARTIN, M. G., MCKAY, C., MING, D. W., NAVARRO-GONZÁLEZ, R., PAVLOV, A., STEELE, A., SUMMONS, R. E., SZOPA, C., TEINTURIER, S. & MAHAFFY, P. R. 2013. Evidence for perchlorates and the origin of

chlorinated hydrocarbons detected by SAM at the Rocknest aeolian deposit in Gale Crater. *Journal of Geophysical Research: Planets,* 118**,** 1955-1973.

GOORDIAL, J., ALTSHULER, I., HINDSON, K., CHAN-YAM, K., MARCOLEFAS, E. & WHYTE, L. G. 2017. In Situ Field Sequencing and Life Detection in Remote (79 degrees 26'N) Canadian High Arctic Permafrost Ice Wedge Microbial Communities. *Front Microbiol,* 8**,** 2594.

GUPTA, A., GUPTA, R. & SINGH, R. L. 2017. Microbes and Environment. *Principles and Applications of Environmental Biotechnology for a Sustainable Future.*

HARTMANN, F. S. F., ANASTASIOU, I., WEISS, T., SHEN, J. & SEIBOLD, G. M. 2021. Impedance flow cytometry for viability analysis of Corynebacterium glutamicum. *J Microbiol Methods,* 191**,** 106347.

HORNE, W. H., VOLPE, R. P., KORZA, G., DEPRATTI, S., CONZE, I. H., SHURYAK, I., GREBENC, T., MATROSOVA, V. Y., GAIDAMAKOVA, E. K., TKAVC, R., SHARMA, A., GOSTINCAR, C., GUNDE-CIMERMAN, N., HOFFMAN, B. M., SETLOW, P. & DALY, M. J. 2022. Effects of Desiccation and Freezing on Microbial Ionizing Radiation Survivability: Considerations for Mars Sample Return. *Astrobiology,* 22**,** 1337-1350.

JAVAUX, E. J. 2006. Extreme life on Earth--past, present and possibly beyond. *Res Microbiol,* 157**,** 37-48.

JONES, E. G., LINEWEAVER, C. H. & CLARKE, J. D. 2011. An extensive phase space for the potential martian biosphere. *Astrobiology,* 11**,** 1017-33.

KOUNAVES, S. 2007. Life on Mars may be hidden like Earth's extremophiles. *Nature,* 449**,** 281.

LASKAR, J., CORREIA, A. C. M., GASTINEAU, M., JOUTEL, F., LEVRARD, B. & ROBUTEL, P. 2004. Long term evolution and chaotic diffusion of the insolation quantities of Mars. *Icarus,* 170**,** 343-364.

LEVER, M. A., ALPERIN, M., ENGELEN, B., INAGAKI, F., NAKAGAWA, S., STEINSBU, B. O. & TESKE, A. 2006. Trends in Basalt and Sediment Core Contamination During IODP Expedition 301. *Geomicrobiology Journal,* 23**,** 517-530.

LEVIN, G. V. 1972. Detection of metabolically Produced labeled gas: The Viking Mars Lander. *Icarus,* 16**,** 153-166.

LEVIN, G. V. & STRAAT, P. A. 2016. The Case for Extant Life on Mars and Its Possible Detection by the Viking Labeled Release Experiment. *Astrobiology,* 16**,** 798-810.

MADIGAN, M. T., BENDER, K. S., BUCKLEY, D. H., STAHL, D. A. & SATTLEY, W. M. 2017. *Brock Biology of Microorganisms, Books a la Carte Edition*, Pearson Education.

MCFARLAND, J. 1907. The Nephelometer:An Instrument for Estimating the Number of Bacteria in Suspensions Used for Calculating the Opsonic Index and for Vaccines. *JAMA: The Journal of the American Medical Association,* XLIX.

MUSILOVA, M., WRIGHT, G., WARD, J. M. & DARTNELL, L. R. 2015. Isolation of Radiation-Resistant Bacteria from Mars Analog Antarctic Dry Valleys by Preselection, and the Correlation between Radiation and Desiccation Resistance. *Astrobiology,* 15**,** 1076-90.

NARESH, V. & LEE, N. 2021. A Review on Biosensors and Recent Development of Nanostructured Materials-Enabled Biosensors. *Sensors (Basel),* 21.

PENDLETON, Y. J. & ALLAMANDOLA, L. J. 2002. The Organic Refractory Material in the Diffuse Interstellar Medium: Mid-Infrared Spectroscopic Constraints. *The Astrophysical Journal Supplement Series,* 138**,** 75-98.

PICARD, C., PONSONNET, C., PAGET, E., NESME, X. & SIMONET, P. 1992. Detection and enumeration of bacteria in soil by direct DNA extraction and polymerase chain reaction. *Appl Environ Microbiol,* 58**,** 2717-22.

PIZZARELLO, S. 2007. The chemistry that preceded life's origin: a study guide from meteorites. *Chem Biodivers,* 4**,** 680-93.

POGHOSYAN, A. & GOLKAR, A. 2017. CubeSat evolution: Analyzing CubeSat capabilities for conducting science missions. *Progress in Aerospace Sciences,* 88**,** 59-83.

PUSPITA, I. D., KAMAGATA, Y., TANAKA, M., ASANO, K. & NAKATSU, C. H.

2012. Are uncultivated bacteria really uncultivable? *Microbes Environ,* 27**,** 356-66.

QUINN, R., GRUNTHANER, P., TAYLOR, C., BRYSON, C. & GRUNTHANER, F. The radiolytic decomposition of soil perchlorates on Mars. 42nd Annual Lunar and Planetary Science Conference, 2011. 2003.

SMITH, D. C., SPIVACK, A., FISK, M. R., HAVEMAN, S. A. & STAUDIGEL, H. 2000. Methods for quantifying potential microbial contamination during deep ocean coring. *ODP Technical Note,* 28**,** 1-17.

SUMMONS, R. E. 1993. Biogeochemical Cycles. *Organic Geochemistry.*

SUMMONS, R. E., AMEND, J. P., BISH, D., BUICK, R., CODY, G. D., DES MARAIS, D. J., DROMART, G., EIGENBRODE, J. L., KNOLL, A. H. & SUMNER, D. Y. 2011. Preservation of martian organic and environmental records: final report of the Mars biosignature working group. *Astrobiology,* 11**,** 157-81.

SWINDLE, T. D., ATREYA, S., BUSEMANN, H., CARTWRIGHT, J. A., MAHAFFY, P., MARTY, B., PACK, A. & SCHWENZER, S. P. 2022. Scientific Value of Including an Atmospheric Sample as Part of Mars Sample Return (MSR). *Astrobiology,* 22**,** S165-S175.

VAGO, J. L., WESTALL, F., COATES, A. J., JAUMANN, R., KORABLEV, O., CIARLETTI, V., MITROFANOV, I., JOSSET, J. L., DE SANCTIS, M. C., BIBRING, J. P., RULL, F., GOESMANN, F., STEININGER, H., GOETZ, W., BRINCKERHOFF, W., SZOPA, C., RAULIN, F., WESTALL, F., EDWARDS, H. G. M., WHYTE, L. G., FAIREN, A. G., BIBRING, J. P., BRIDGES, J., HAUBER, E., ORI, G. G., WERNER, S., LOIZEAU, D., KUZMIN, R. O., WILLIAMS, R. M. E., FLAHAUT, J., FORGET, F., VAGO, J. L., RODIONOV, D., KORABLEV, O., SVEDHEM, H., SEFTON-NASH, E., KMINEK, G., LORENZONI, L., JOUDRIER, L., MIKHAILOV, V., ZASHCHIRINSKIY, A., ALEXASHKIN, S., CALANTROPIO, F., MERLO, A., POULAKIS, P., WITASSE, O., BAYLE, O., BAYON, S., MEIERHENRICH, U., CARTER, J., GARCIA-RUIZ, J. M., BAGLIONI, P., HALDEMANN, A., BALL, A. J.,

DEBUS, A., LINDNER, R., HAESSIG, F., MONTEIRO, D., TRAUTNER, R., VOLAND, C., REBEYRE, P., GOULTY, D., DIDOT, F., DURRANT, S., ZEKRI, E., KOSCHNY, D., TONI, A., VISENTIN, G., ZWICK, M., VAN WINNENDAEL, M., AZKARATE, M. & CARREAU, C. 2017. Habitability on Early Mars and the Search for Biosignatures with the ExoMars Rover. *Astrobiology,* 17**,** 471-510.

WADSWORTH, J. & COCKELL, C. S. 2017. Perchlorates on Mars enhance the bacteriocidal effects of UV light. *Sci Rep,* 7**,** 4662.

WESTALL, F., FOUCHER, F., BOST, N., BERTRAND, M., LOIZEAU, D., VAGO, J. L., KMINEK, G., GABOYER, F., CAMPBELL, K. A., BREHERET, J. G., GAUTRET, P. & COCKELL, C. S. 2015. Biosignatures on Mars: What, Where, and How? Implications for the Search for Martian Life. *Astrobiology,* 15**,** 998-1029.

WILHELM, M. B., DAVILA, A. F., PARENTEAU, M. N., JAHNKE, L. L., ABATE, M., COOPER, G., KELLY, E. T., PARRO GARCIA, V., VILLADANGOS, M. G., BLANCO, Y., GLASS, B., WRAY, J. J., EIGENBRODE, J. L., SUMMONS, R. E. & WARREN-RHODES, K. 2018. Constraints on the Metabolic Activity of Microorganisms in Atacama Surface Soils Inferred from Refractory Biomarkers: Implications for Martian Habitability and Biomarker Detection. *Astrobiology,* 18**,** 955-966.

YAMAGISHI, A., YOSHIMURA, Y., HONDA, H., MIYAKAWA, A., SATOH, T., ISHIGAMI, G., HARUYAMA, J., KOBAYASHI, K., NAGANUMA, T., SASAKI, S. & MIYAMOTO, H. 2014. MELOS Life Search Plan: Search for Microbes on the Mars Surface with Special Interest in Methane-oxidizing Bacteria. *Transactions of the Japan Society for Aeronautical and Space Sciences, Aerospace Technology Japan,* 12**,** Tk_41-Tk_48.

964ZHANG, L., ZENG, F., MCKAY, C. P., NAVARRO-GONZALEZ, R. & SUN, H. J. 2021. Optimizing Chiral Selectivity in Practical Life-Detection Instruments. *Astrobiology,* 21**,** 505-510.

Appendix

Scientific communications

Publications:

- Yongda Li, David A. Collins, and Konstantinos Grintzalis. " Development of Sensitive Methods for the Detection of Minimum Concentrations of DNA on Martian Soil Simulants." *Life* 13.10 (2023): 1999.
- Yongda Li, David A. Collins, and Konstantinos Grintzalis. "A Simple Biochemical Method for the Detection of Proteins as Biomarkers of Life on Martian Soil Simulants and the Impact of UV Radiation." *Life* 13.5 (2023): 1150.
- Yongda Li, David A. Collins, and Konstantinos Grintzalis. "A simple approach for the detection of Escherichia coli as a model bacterium on Martian soil simulants: A proof of concept study." *Journal of Microbiological Methods* (2023): 106751.

Conferences:

- Yongda Li, David Collins, Konstantinos Grintzalis. (2019). Simple biochemical methods for the detection of life-inhibiting peroxidants and life signatures on Mars-like soils. European Astrobiology Network Association 19th EANA Astrobiology Conference (Orléans, France). Oral Presentation.
- Yongda Li, David Collins, Konstantinos Grintzalis. (2020). Development of simple-step, ultrasensitive protein detection assays for Mars-like soils. Molecular Origins of Life, Munich 2020 (Online). Posters.
- Yongda Li, David Collins, Konstantinos Grintzalis. (2020). Development of simple-step, ultrasensitive protein detection assays for Mars-like soils. Astrochemical Frontiers – Quarantine Edition 2020 (Online). Posters.
- Yongda Li, David Collins, Konstantinos Grintzalis. (2020). A biochemical method for the detection of proteins as life signatures on Mars-like soils. European Astrobiology Network Association EANA 2020 Virtual Conference (Online). Oral Presentation, Space Factor Contest.

- Yongda Li, David Collins, Konstantinos Grintzalis. (2020). Development of simple-step, ultrasensitive protein detection assays for Mars-like soils. Astrobiology Australasia Meeting 2020 (Online). Posters.
- Yongda Li, David Collins, Konstantinos Grintzalis. (2021). A biochemical method for the detection of proteins as life signatures on Mars-like soils. The Biological Research Society 12th Annual Research Day (Online). Oral Presentation, won the award for Highly Commended.
- Yongda Li, David Collins, Konstantinos Grintzalis. (2021). A simple approach for the detection of microorganisms on Martian soil simulants. The Origins 2021 Online Conference (Online). Posters.
- Yongda Li, David Collins, Konstantinos Grintzalis. (2021). A simple approach for the detection of microorganisms on Martian soil simulants. The Virtual 5th NoRCEL Conference (Online). Posters.
- Yongda Li, David Collins, Konstantinos Grintzalis. (2021). A simple approach for the detection of microorganisms on Martian soil simulants. Molecular Origins of Life, Munich 2021 (Online). Posters.
- Yongda Li, David Collins, Konstantinos Grintzalis. (2021). A simple approach for the detection of microorganisms on Martian soil simulants. European Astrobiology Network Association EANA 2021 Virtual Conference (Online). Poster Presentation.
- Yongda Li, David Collins, Konstantinos Grintzalis. (2022). Searching for microorganisms as life signals for planetary missions. The Microbiology Society Annual Conference 2022 April 4-5 (Belfast, UK). Poster Presentation.
- Yongda Li, Keith D Rochfort, David Collins, Konstantinos Grintzalis. (2022). Searching for DNA as biosignatures of extra-terrestrial life for planetary missions. COSPAR 2022 - 44th Scientific Assembly. July 16-24 (Athens, Greece). Poster Presentation.

Workshops:

- Yongda Li. EANA International Spring School 2021 on hydrothermal vents. May 17-21, 2021. Virtual. EANA Spring School organizing committee. Certificate of completion.
- Yongda Li. Workshop on Terrestrial Analogs for Planetary Exploration. June 16-18, 2021. Virtual. USGS Astrogeology Science Center.
- Yongda Li. Workshop on RED'21 Astrobiology Introductory Course. June 21-26, 2021. Virtual. RED'21. Certificate of completion.
- Yongda Li. Workshop for the microsensor training. September 13-16, 2021. Virtual. Unisense.
- Yongda Li. The oLife plenary talks (The oLife Fellowship programme Annual Meeting 2021). November 11-12, 2021. Virtual. oLife Cross-Networking committe.
- Yongda Li. Workshop on RED'22 Astrobiology Introductory Course. March 13-19, 2022. Le Teich, France. Certificate of completion.